



University of Kentucky
UKnowledge

Theses and Dissertations--Chemical and
Materials Engineering

Chemical and Materials Engineering

2011

STABILITY OF AFFINITY BASED LAYER-BY-LAYER POLYMERIC SELF-ASSEMBLIES FOR ORAL WOUND APPLICATIONS

Sundar Prasanth Authimoolam

University of Kentucky, sundar.prasanth@uky.edu

[Right click to open a feedback form in a new tab to let us know how this document benefits you.](#)

Recommended Citation

Authimoolam, Sundar Prasanth, "STABILITY OF AFFINITY BASED LAYER-BY-LAYER POLYMERIC SELF-ASSEMBLIES FOR ORAL WOUND APPLICATIONS" (2011). *Theses and Dissertations--Chemical and Materials Engineering*. 3.

https://uknowledge.uky.edu/cme_etds/3

This Master's Thesis is brought to you for free and open access by the Chemical and Materials Engineering at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Chemical and Materials Engineering by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained and attached hereto needed written permission statements(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine).

I hereby grant to The University of Kentucky and its agents the non-exclusive license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless a preapproved embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's dissertation including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Sundar Prasanth Authimoolam, Student

Dr. Thomas D. Dziubla, Major Professor

Dr. Stephen Rankin, Director of Graduate Studies

STABILITY OF AFFINITY BASED LAYER-BY-LAYER POLYMERIC SELF-ASSEMBLIES FOR ORAL
WOUND APPLICATIONS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Chemical Engineering in the College of Engineering
at the University of Kentucky

By

Sundar Prasanth Authimoolam

Lexington, Kentucky

Director: Dr. Thomas D. Dziubla, Assistant Professor of Chemical Engineering

Co-Director: Dr. David A. Puleo, Professor of Biomedical Engineering

Lexington, Kentucky

2011

Copyright © Sundar Prasanth Authimoolam 2011

ABSTRACT OF THESIS

STABILITY OF AFFINITY BASED LAYER-BY-LAYER POLYMERIC SELF-ASSEMBLIES FOR ORAL WOUND APPLICATIONS

Oral mucositis is a painful and debilitating chronic inflammatory condition that can result from chemo and/or radiotherapy. While current treatment strategies which provide temporary relief exist, there is still an unmet clinical need for a robust long active barrier strategy which can simultaneously provide protection and release drug to enhance the wound healing response. It is proposed that an affinity based layer-by-layer self-assembled barrier administered as a series of mouth rinses can allow for wound specific drug delivery, providing an effective regenerative therapy.

In this work, biotinylated poly(acrylic acid) is used to develop LBL assemblies based upon biotin-streptavidin affinity interactions. To explore the ability of developed LBL assemblies to resist the harsh intraoral environment, *in vitro* chemical and *ex vivo* mechanical tests are performed. The stability results demonstrate significant LBL barrier stability with wear resistance. From principal component regression analysis, factors such as polymer MW and number of layers in assemblies contributed significantly to chemical barrier stability. Also it is observed that the extent of biotin conjugation plays a significant role in LBL development and in mechanical stability. Thus, the proposed affinity based multilayered assemblies with their excellent barrier properties offer a modular treatment approach in oral mucosal injuries.

KEYWORDS: Oral mucositis, Biotin-Streptavidin, Layer-by-Layer, Poly (acrylic acid), Oral drug delivery

Sundar Prasanth Authimoolam

November 28, 2011

STABILITY OF AFFINITY BASED LAYER-BY-LAYER POLYMERIC SELF-ASSEMBLIES FOR ORAL
WOUND APPLICATIONS

By

Sundar Prasanth Authimoolam

Dr. Thomas D. Dziubla

Director of Thesis

Dr. Stephen Rankin

Director of Graduate Studies

November 28, 2011

Date

DEDICATION

(To my ever caring parents)

ACKNOWLEDGEMENTS

I would like to take this as an opportunity in expressing sincere gratitude for my advisor, Dr. Thomas Dziubla. All the research skills which I gained during this master's study should be credited to my advisor, to say the least. His patience and his efforts to motivate me were truly commendable. I am grateful for his support and his constant guidance without which this work would not have been possible. I would like to thank my co-advisor Dr. David Puleo for his support in mechanical testing studies and acknowledge him for his insightful suggestions. I acknowledge Paritosh P. Wattamwar, friend and my fellow lab-mate for helping me train and quicker learn the available analytical equipments in our lab. I thank Sandeep K. Ramineni from Biomedical Engineering, for his help and assistance while performing Bose adhesion testing studies. I would also like to thank my committee member, Dr. Stephen Rankin for his assistance in my defense process. Finally, I acknowledge all my friends and lab mates (Dr. Dziubla's Biomaterial group), who made my stay in Lexington memorable.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
CHAPTER 1. INTRODUCTION	1
CHAPTER 2. BACKGROUND	3
2.1. Problem Statement	3
2.2. Risk factors	4
2.2.1. Treatment related factors	4
2.2.2. Patient related factors.....	5
2.3. Pathobiology of oral mucositis.....	6
2.4. Oral mucositis Assessment Scales.....	10
2.5. Barriers in prevention or treatment of oral mucositis.....	11
2.6. Preventive Strategies	12
2.7. Current treatment Strategies.....	13
2.8. Proposed Modular Treatment Strategy	15
CHAPTER 3. RESEARCH GOALS	27
3.1. Objective/Hypothesis.....	27
3.1.1. Specific aim #1: Synthesize and characterize biotin functionalized polymers.....	27
3.1.2. Specific aim #2: Formulate LBL self-assemblies and evaluate <i>in vitro</i> chemical stability.	27
3.1.3. Specific aim #3: Evaluate LBL mechanical stability through <i>ex vivo</i> adhesion studies.....	28
CHAPTER 4. POLY(ACRYLIC ACID) BIOTINYLATION AND CHARACTERIZATION.....	29

4.1. Materials and methods	30
4.1.1. Materials.....	30
4.1.2. Polymer biotinylation synthesis	30
4.1.3. Purification and characterization	31
4.2. Results and discussion.....	32
4.2.1. Polymer biotinylation	32
CHAPTER 5. CHEMICAL STABILITY TESTS ON LAYER-BY-LAYER SELF-ASSEMBLIES	39
5.1. Materials and methods	40
5.1.1. Developing affinity based Layer by Layer (LBL) assemblies	40
5.1.2. Chemical stability tests on <i>in vitro</i> LBL assemblies	42
5.1.3. Statistical analysis.....	42
5.1.4. LBL degradation mechanism	43
5.2. Results and Discussion	44
5.2.1. Developing Layer-by-Layer self-assemblies.....	44
5.2.2. LBL chemical stability.....	48
5.2.3. Statistical analysis on LBL chemical stability	51
5.2.4. LBL degradation mechanism	51
Chapter 6. MECHANICAL STABILITY TESTS ON LAYER-BY-LAYER SELF-ASSEMBLIES	79
6.1. Materials and methods	79
6.1.1. <i>Ex vivo</i> LBL.....	79
6.1.2. Mechanical stability tests on <i>ex vivo</i> LBL assemblies	79
6.2. Results and Discussion	80
6.2.1. <i>Ex vivo</i> LBL.....	80
6.2.2. LBL mechanical stability.....	81

CHAPTER 7. CONCLUSION AND FUTURE STUDIES.....	88
APPENDIX.....	89
LIST OF ABBREVIATIONS	109
REFERENCES.....	111
VITA.....	124

LIST OF TABLES

Table 2-1: Evaluating severity of oral mucositis - various scoring methods with their employed method of assessment.....	17
Table 2-2: Use of non-therapeutic method as a prophylactic strategy in OM.....	19
Table 2-3: Use of topical analgesics as a prophylactic strategy in OM.	20
Table 2-4: Use of various biology response modifiers as a prophylactic strategy in OM.	21
Table 2-5: Use of various oral mouth rinses as a treatment strategy in OM.	22
Table 2-6: Use of Mucoadhesive gels as a treatment strategy in OM.	23
Table 5-1: Table shows summarized results of PCR analysis on <i>in vitro</i> LBL assembly development.....	54
Table 5-2: Summarized results from 3-way ANOVA model (Generalized Linear Model) on main effects of <i>in vitro</i> LBL assembly development.	55
Table 5-3: Summarized results of principal component regression (PCR) analysis on LBL protease stability response variables	56
Table 5-4: Summarized results of principal component regression (PCR) analysis on LBL salivary stability response variables	57
Table 5-5: Summarized results from 3-way nested ANOVA model on LBL chemical stability.....	58
Table 5-6: Summary of results from 3-way ANOVA models and from post-hoc analysis.	59

LIST OF FIGURES

Figure 2-1: Different grades of oral mucositis as per NCI assessment scales.	24
Figure 2-2: Scheme showing pathogenesis of oral mucositis, potential treatment strategies and treatment barriers associated with oral mucosal wounds.	25
Figure 2-3: Scheme showing overall proposed application of LBL self- assemblies in oral drug delivery.	26
Figure 4-1: Synthesis scheme for poly(acrylic acid) biotinylation.	34
Figure 4-2: Reverse phase HPLC for detecting removal of unreacted biotin from reaction sample.	35
Figure 4-3: Scheme showing mechanism of HABA-avidin analysis	36
Figure 4-4: UV-visible spectra of HABA-based assays for biotin. Extent of biotinylation is determined through free HABA release	37
Figure 4-5: HABA analysis results showing extent of polymer biotinylation.	38
Figure 5-1: Extent of base layer coverage - streptavidin adsorption on base layer.....	60
Figure 5-2: Scheme showing LBL assembly development using biotin-streptavidin affinity linkages	61
Figure 5-3: <i>In vitro</i> LBL assembly formation using high MW (90,000 Da) PAA of various extent of biotin conjugation.	62
Figure 5-4: <i>In vitro</i> LBL assembly formation using mid-range MW (50,000 Da) PAA of various extent of biotin conjugation.....	63

Figure 5-5: <i>In vitro</i> LBL assembly formation using low MW (10,000 Da) PAA of various extent of biotin conjugation.	64
Figure 5-6: Main effects plot from ANOVA studying LBL factor effects on development of <i>in vitro</i> LBL assemblies.	65
Figure 5-7: Interactions plot from ANOVA studying LBL factor effects on development of <i>in vitro</i> LBL assemblies.	66
Figure 5-8: Effect of factor “extent of biotin conjugation” in LBL protease stability.	67
Figure 5-9: Effect of factor “number of assembly layers (NoL)” in LBL protease stability.	68
Figure 5-10: Effect of factor “polymer MW” in LBL protease stability.....	69
Figure 5-11: Streptavidin mass loss (%) at M_{∞} in LBL protease stability.....	70
Figure 5-12: Effect on factor “extent of biotin conjugation” in LBL salivary stability.	71
Figure 5-13: Effect on factor “number of assembly layers (NoL)” in LBL salivary stability.	72
Figure 5-14: Effect on factor “polymer MW” in LBL salivary stability.....	73
Figure 5-15: Streptavidin mass loss (%) at M_{∞} in LBL salivary stability.....	74
Figure 5-16: Scheme showing design used in 3-way nested ANOVA analysis	75
Figure 5-17: Possible LBL degradation mechanism under oral chemical effects	76
Figure 5-18: Scheme shows developed 5-layered LBL systems, to study degradation mechanism.....	77
Figure 5-19: Results studying mechanism of <i>in vitro</i> LBL degradation.	78
Figure 6-1: <i>Ex vivo</i> tissue LBL assembly formation.	84

Figure 6-2: Scheme for <i>ex vivo</i> LBL adhesion testing.	85
Figure 6-3: Repeated contact barrier fatigue tests.	86
Figure 6-4: Summary of results from mechanical testing.	87

CHAPTER 1. INTRODUCTION

Oral mucositis (OM), an inflammatory ulcerous oral wound condition, is a commonly occurring side-effect of anti-cancer therapies, including chemotherapy and/or radiotherapy [1]. This debilitating acute pathology causes severe pain leading to dysphasia and impacts patients overall quality of life [2-4]. Every year in the United States, approximately 132,000 patients undergoing anti-cancer therapies develop OM (as per 2003 data) [5, 6]. More than 90% of patients subjected to head and neck cancer therapies (HNC) develop OM, with the estimated cost per patient exceeding \$17,000 (USD) [4, 7-9].

Current strategies employed in OM treatment are similar to methods used for dermatological cases such as oral gels or lubrication mouth rinses that can provide only a temporary palliative relief with poor patient compliance [6, 10]. In order to overcome the inadequacies in current treatment methods, a modular treatment strategy is proposed utilizing biotin-streptavidin affinity linkages for developing multilayered polymeric self-assemblies. It is hypothesized that a targeted layer-by-layer (LBL) polymeric self-assembly developed over the oral wound surface will offer a desired regenerative treatment strategy through its stable barrier effects. Hence, evaluating the barrier stability of LBLs against the harsh intraoral environment is a key requirement to validate its application in oral wounds.

In this work, *in vitro* chemical stability tests are performed by studying the proteolytic effects of unstimulated whole saliva (UWS) and protease (pronase) on LBL

assemblies. Through chemical stability tests, destabilization of self-assembled layers is evaluated. Statistical analyses (multivariate analysis and ANOVA models) are performed to identify key factors (polymer molecular weight / biotin conjugation / number of assembly layers), contributing to LBL chemical stability. Developing an understanding of the key factors that control LBL chemical stability is instrumental in formulating a tunable LBL system with desired barrier properties. In order to study LBL barrier durability and wear resistance, *ex vivo* mechanical tests (adhesion tests) are performed. Porcine patches are used as a model tissue substrate for developing LBL assemblies in adhesion tests. A repeat contact barrier fatigue test is performed on LBL developed on tissue substrate to determine the extent of LBL barrier loss (wear resistance) from repeated loading cycles (load-pull offs).

CHAPTER 2. BACKGROUND

2.1. Problem Statement

Oral mucositis, a painful and debilitating oral wound condition, is a frequently occurring post-operative complication in anti-cancer therapies such as radiotherapy, chemotherapy, chemoradiotherapy and hematopoietic stem cell transplants (HSCT) [11-14]. OM is manifested by erythema and inflammatory lesions, which rupture through the oral epithelial mucosal walls [1, 15]. This mucosal barrier injury causes severe pain with loss of oral functionality, thereby resulting in reduced nutritional uptake and dysphasia [4, 16, 17].

OM compromises the patient's overall quality of life by not only affecting routine functions, such as eating, swallowing or speaking but also through its high cost for treatment and care [18]. Invariably, affected patients experience an increased duration of hospital care, adding to the economic burden of their treatment. This was exemplified in literature studies[19, 20], which showed that patients with OM induced by autologous transplants required an additional hospitalization time of at least 5 days, which contributed to an expenditure of \$22,500/ patient. A study conducted by Sonis et al. [21] about economic outcomes of OM in HSCT patients report a total hospital charge of \$42,479 higher than patients who do not develop OM. Also, in patients suffering from OM, there is an increased risk of infection through colonization of bacterial flora, which aggravates the existing oral wound condition. Finally, OM can interrupt cancer treatment, a result of apoptotic response and cellular death from radiation or

chemotherapeutic doses. This affects patient's treatment regime and possess a potential risk of death from treatment delays [22].

Despite such serious effects of OM and its impact in patients life, the use of potent anti-cancer therapies and administrating aggressive treatment regimes has become an inescapable part of cancer treatment and control [23]. Hence, it is unsurprising to observe high incidence rates of OM, where nearly 30%-75% patients subjected to chemotherapy and 70% - 90% of bone marrow recipients are affected [1, 13, 17]. The incidence rate of OM was found to be even more for head and neck radiotherapy, greater than 90 % [4, 7].

2.2. Risk factors

The incidence of OM and its severity can be described by two broad categories, treatment related and patient related factors. Subcategories under treatment related factors include type of cancer treated, treatment method employed, dosage, and treatment regimes. In patient related factors, variables such as oral hygiene, age and gender can affect OM incidence, severity and the disease duration [1, 20, 24].

2.2.1. Treatment related factors

Among treatment factors, the risk among cancer types is highly dependent on the zone of treatment, where cancer treatment involving head and neck regions have been found to be more susceptible to development of ulcerous lesions. In contrast, a relatively low risk is found among treatments in other regions [25]. Also, the incidence of severe OM is frequent among patients subjected to conditioning therapy after bone marrow transplants (>60%) [20, 26-28].

Interestingly, from a study of 150 patients, there is a reported high frequency (1 in 3 incidence) of mucositis (mild to severe) due to the chemotherapeutic effect on solid tumors, emphasizing the potential treatment impact [25]. Among patients subjected to high dose chemo/radiotherapy regimes, the incidence rate of OM and its extent of severity increases. Repeated low chemotherapeutic dosages also possess a high risk as when compared to bolus infusion. Hence, apart from dosage factors the number of cycles that was previously administered also affect the risk of OM incidence[28].

In radiotherapy induced OM, various factors such as irradiated mucosal volume, dose intensity, applied cumulative dosage and other dietary factors such as smoking or drinking prior to treatment can have an impact on disease severity and its duration [28-30]. For the standard dose of 200 centi-Gray/day onset of erythema can be seen within a week of treatment, which on accumulating cumulative total dose of 1600-2000 cGy will result in atrophy of oral epithelial walls [28, 31-33]. Such irradiations can also damage salivary glands, resulting in xerostomia (hyposalivation), where the resulting oral dryness worsens the existing condition due to lack of essential salivary functions such as oral lubrication, regulation of acidity and mucosa protection [33, 34].

2.2.2. Patient related factors

In patient related factors, the existing wound condition can be exacerbated by maintaining poor oral hygiene, which causes excessive oral flora and increased infection rate [17, 33, 35]. Age also impacts the rate of OM. The frequency of OM incidence is higher among younger people (<20 years), a result of greater number of rapidly dividing mitotic cells (basal cell proliferation) [15, 33]. In older people (age >50), the developed

mucositis is more severe, necessitating greater healing time[15]. OM incidence during chemotherapy is also increased among patients with poor nutritional status or reduced food uptake during the treatment regime. Interestingly, patients with low body mass index (BMI<20) were found to be more susceptible to developing OM [25, 36].

2.3. Pathobiology of oral mucositis

The development of OM can be broken down into 5 key stages, as described by Sonis et. al., [37].

- 1) Initiation
- 2) Primary damage response (Upregulation and message generation)
- 3) Signal amplification
- 4) Ulceration and
- 5) Healing

It should be noted that while these stages are conceptually separated for ease of understanding, the pathological process in OM is dynamic, where the phases can be overlapping depending upon the adopted treatment procedure[38]. For instance, during radiotherapy when patients receive small doses for an extended duration, they invariably develop chronic damage which results in staged overlaps. In contrast, patients subjected to chemotherapy receive short and intensive bolus injections resulting in an acute damage with relatively better distinguished stages [37].

- 1) Initiation

Initiation is the inflammatory phase, also commonly referred to as “initial tissue injury phase”, whose onset occurs rapidly after administering chemotherapy

and/or radiotherapy [13, 37]. This stage is predominately marked by the generation of reactive oxygen species (ROS), resulting in oxidative stress. Here, it is not the initial direct tissue damage (DNA and non-DNA damage) but the generation of ROS that results in extensive tissue damage [39]. This oxidative stress upregulates inflammatory signaling molecules (e.g. nuclear factor- κ B) and biological pathways, and leads to primary damage response which contributes to mucosal damage [4, 37].

2) Primary damage response

In a series of cascading events, ROS activates various transcription factor and multiple pathways, such as nuclear factor- κ B (NF- κ B), STAT3 (signal transducer and activator of transcription 3), p53 (tumor protein 53), and the ceramide pathway [13, 37, 38, 40]. Ceramides (sphingolipids in the form N-acylated sphingosine) were found to be abundant in cell membranes which in response to cellular stress activates several enzymes (e.g. cathepsin D, serine protein phosphatases). Such activation regulates different pathways eventually leading to growth inhibition or cellular death [40].

NF- κ B is considered a central player with its ability to upregulate ~200 genes, whose expression can modulate cytokines production, cell adhesion molecules, stress response genes and cyclooxygenase-2 pathway [38, 41]. NF- κ B, effects both pro-apoptotic and anti-apoptotic response, greatly influencing the fate of normal tissues and effectiveness of cancer treatment [37].

Such upregulation of genes due to transcription factor activation induces production of pro-inflammatory cytokines, including tumor-necrosis factors (TNF-), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-1 β (IL-1 β) [37, 39]. The effect of pro-

inflammatory cytokines along with apoptotic response from ceramide pathway activation (a result of cellular stress from ROS generation) brings forth epithelial damage and injury [37, 40]. Also, studies reported by Bamba et al [42], showed an upregulated effect of cytokines on activator protein-1 (AP-1), which activates matrix metalloproteinases (MMPs) and contributes to tissue injury and inflammation [37, 43]. This effect was pronounced within submucosa, where MMPs disrupt the sub-epithelium (due to MMP1, an interstitial/fibroblast collagenase) and epithelium basement (due to MMP3, also called as Stromelysin-1) resulting in injury [37, 39].

3) Signal amplification

While cytokine production and pathway activation result in primary tissue damage, such effects are further increased due to a signal amplification mechanism. In signal amplification, the pro-inflammatory cytokines or protein from biological pathways stimulate a positive feedback loop response, which increases the impact of primary tissue injury [8, 39]. Such feedback mechanisms [44] follow a complex series of interlinked networks. One such pro-inflammatory cytokine (TNF-) amplifies NF- κ B production, which in turn can upregulate various genes to produce more cytokines or activate pathways to result in injury [38, 39]. As an illustration of the pathway of activation, activated NF- κ B results in more cytokines production, which affects ceramide pathways and results in production of more MMPs, causing an eventual apoptotic response [4, 39].

4) Ulceration

As a result of loss of cells due to the apoptotic effects of preceding events, the oral mucosal epithelium thins out, resulting in an ulcerative stage [4, 45]. For example, during administration of chemotherapeutic doses, the onset of such ulcerous lesion can usually be found within 14 days [45]. This mucosal barrier injury aided by lack of epithelial proliferation becomes a zone of increased bacterial growth[39]. In response to this increased bacterial growth and inflammation, there is an accompanying increase in macrophages and neutrophils infiltration which further augments inflammation and risk of septicemia (sepsis, bacteremia) [4, 8, 39]. Ruescher et al.,[46] illustrated the increased incidence of bacteremia among OM patients undergoing autologous HSCT, where there was an increase in *streptococci* infections. In addition, the cell wall products released from such bacterial floras stimulates the production of pro-inflammatory cytokines through immune pathways [4, 8]. Such release of cytokines, contributes to existing signal amplification process which further debilitates the existing wound condition by aggravating pain and injury.

5) Healing

The healing process commonly occurs within 2-3 weeks following the post-treatment (chemotherapy) process [47]. During the healing phase, down-regulation of the inflammatory response is accompanied by extracellular matrix (ECM) signaling to epithelial cells. ECM contains a complex structural network (fibrous protein, proteoglycans and glycoproteins) which contributes to structural support of tissues [48]. Mesenchyme and ECM signal epithelial migration, proliferation and differentiation which allows for epithelial wound healing [39]. Although such migrations help in

remodeling superficial epithelial injuries, a complete recovery in zones of severe necrosis or deep wounds require a combined effect with angiogenesis [45, 49]. Thus, the duration of the healing phase can be highly dependent upon severity of oral mucositis which in turn is a function of treatment and patient related factors (risk factors).

2.4. Oral mucositis Assessment Scales

Currently, the severity of OM is assessed using several scales including the World Health Organization (WHO), National Cancer Institute (NCI), NCI Common Toxicity Criteria (NCI CTC), Oral Mucositis Assessment Scale (OMAS) and Western Consortium for Cancer Nursing Research (WCCNR) [1, 50]. Such scoring methods have been developed which consider 1.) extent of functionality loss (e.g. dysphasia) 2.) patient's subjective factors (e.g. pain) and/or 3.) objective clinical indicators (e.g. infection) [51]. Detailed biological evaluation employed for each of these scoring methods is listed in **Table 2-1**. Despite the availability of such a variety of scoring methods, there is still a need for commonly followed standard procedures with a capability to meet all desired criteria [17].

To exemplify the debilitating condition of oral mucositis and thereby provide a visual representation of grades involved in one such scoring method, an illustration using NCI assessment scales is shown in **Figure 2-1** (Cawley et.al). The current proposed strategy suggested in this work is expected to offer a potential treatment solution for OM with Grades 2, 3, and 4 (based on the NCI scale). Patients with grade-2 OM exhibit the onset of patchy ulcerations whereas at grade-5 the ulcerations coalesce thereby denude the mucosa. Despite such varied OM grades (2, 3, and 4), the common aspect

between them is the degree of fibrin exposure resulting from epithelial disruption and ulceration lesions. Such fibrin elements, a key component from exposed oral injury [52, 53] can be targeted by a complimentary fibrin binding peptide “CREKA” functionalized in biotinylated polymer. CREKA (Cys-Arg-Glu-Lys-Ala) is a pentapeptide widely reported in the literature for its effective fibrin targeting ability [54-58]. The targeted CREKA functionalized polymeric layer forms an adhesive base layer over oral pathology. By providing a series of alternating protein streptavidin and biotinylated polymer mouth rinses, multilayered polymeric barrier systems (layer-by-layer (LBL)) can be developed over the targeting layer, as shown in **Figure 2-3**.

2.5. Barriers in prevention or treatment of oral mucositis

One major hindrance in treating OM is due to the conventional belief among health care providers that mucositis is solely a result of damage to the rapidly dividing oral epithelial walls. However, with recent greater understanding of the pathobiological mechanism of OM, there is a huge future potential for developing better pharmacological products utilizing a mechanistic based treatment approach [17, 37, 39, 59]. Such mechanistic based treatment approaches under clinical investigation predominantly function by modifying the biological responses (e.g. keratinocyte growth factor or epidermal growth factor).

With current treatments being similar to those adapted for dermatological cases, there is a need for better redesigned approaches specifically tailored for oral mucosal applications. Oral mucosa exists in a complex environment where they are subjected to continual salivary flushing activity. Although salivary action helps to provide a hydrating

medium for drug distribution, such effects could also result in rapid drug clearance by swallowing [60, 61]. In overcoming such drawbacks, a drug delivery device should be capable of minimizing salivary dilution effects thereby potentially obviating the need for repeated drug dosing [60]. Another major block in developing oral mucosal delivery systems is the harsh intra-oral environment, which characteristically possesses exuding fluids, mastication and proteolytic degradation effects, which present unique challenges in developing an effective drug carrier for periodontal drug delivery [60] (**Figure 2-2**).

2.6. Preventive Strategies

To address OM, a prophylactic strategy would be an ideal treatment method, but in reality such prevention is hardly possible [17, 62]. Prophylactic methods using non-therapeutic methods such as oral cryotherapy (minimizing effect of OM due to chemotherapy), low-intensity lasers, midline radiation blocks, and three-dimensional radiation treatment (to reduce OM from radiation therapy) are often recommended in preventing OM[1, 7]. A more detailed description of various preventive strategies (non-therapeutic methods) is provided in **Table 2-2**.

Recently, the use of topical analgesics is often suggested as an alternative method. The majority of such analgesics function by inhibiting pro-inflammatory cytokine production. Such down-regulation results in pain reduction and brings forth an anti-inflammatory effect. A more detailed review on various topical analgesics is listed in **Table 2-3**.

With recent greater understanding about pathophysiology of OM as described earlier, the use of biological response modifiers in prophylaxis has been widely

investigated. Such prevention was a result of modification in key factors controlling disease severity such as downregulation of cytokine production or upregulating epithelial proliferation and/or positively influencing angiogenesis process. A review of various biological response modifiers is listed in **Table 2-4**. While the use of these prophylactic methods reduced the impact of mucosal injuries, consensus has not been reached on protocols and easily adopted and tolerated delivery strategies are needed before they gain wide acceptance.

2.7. Current treatment Strategies

The majority of the current treatment methods focus on symptom management rather than providing regenerative therapy. Various topical treatment methods, [63] being adopted or under investigation involve formulation such as,

- 1) Oral mouth rinses - e.g. MuGuardTM and Caphosol[®] [64]

- 2) Bioadherent gels - e.g. Gelclair[®] [65]

- 3) Oral sprays

e.g. recombinant human intestinal trefoil factor (rhITF) oral spray [66] and

recombinant human epidermal growth factor (rhEGF) sprays [67]

- 4) Patches - e.g. flurbiprofen tooth patch[68]

- 5) Tablets - e.g. Xylocaine buccal tablets

Oral mouth rinses are quite common as a treatment strategy and are predominantly non-targeted in their mode of treatment. They function by providing a moistening effect, which enables rapid pain relief and prevents oral dryness (Xerostomia). Caphosol[®] (a supersaturated calcium phosphate solution) is an example

of one such oral mouth rinse, which prevents oral dryness through its lubricative effect and thereby offers a temporary palliative effect. Another commonly used oral mouth rinse is “magic mouth wash”, a combination of multifunctional ingredients such as antibiotic, antihistamine, antifungal, steroid, local anesthetic or antacid [69]. This multifunctional formulation was found to be ineffective in treating OM, likely a result of the lack of any targeting and drug localization capacity, and therefore had therapeutic value equivalent to a non-therapeutic saline mouth wash [17, 70, 71]. In treating OM, a few commonly desired therapeutic effects involve: anti-inflammatory, analgesic (e.g. benzydamine), topical anesthetic (e.g. lidocaine) and antimicrobial properties (e.g. chlorhexidine) [3, 59]. Studies evaluating efficacy of various oral mouth rinses with such ingredients either independently or in combination are quite prevalent. A more detailed list on various oral mouth rinses is provided in **Table 2-5**.

Much of the exciting recent work on OM has focused on developing oral bioadhesive films and gels for localized drug delivery [72-74]. Bioadhesive topical films (e.g. hydroxypropyl-cellulose (HPC) based film (Zilactin)) function primarily through their protective barrier effects over oral ulcerations and thereby provide temporary pain relief [3]. Gelclair, a commonly used mucoadherent gel (polyvinylpyrrolidone, sodium hyaluronate formulation), functions by providing a lubricative moistening effect [17]. Although most of the mucoadhesive gels help in providing rapid pain relief, such effects are temporary. A detailed list of different mucoadhesive gels is provided on **Table 2-6**. Unfortunately, patients suffering from OM invariably develop a highly irregular mucosal surface from extensive ulcerous sores (occupying >50% of the oral cavity [3]) making

application of such bulky films and gels difficult, especially along the gumline. Hence, with such current ineffective treatment methods, there is still a clinical need for an improved treatment strategy for oral mucosal disease.

2.8. Proposed Modular Treatment Strategy

To achieve an effective OM therapy, it is desired to develop a strategy which can

1.) specifically target oral wound surfaces, 2.) prevent oral dryness by maintaining a lubricative surface, 3.) effectively provide long term controlled release of anti-inflammatory and wound healing agents, 4.) resist bacterial invasion, 5.) minimize pain and inflammation, 6.) be durable to the chemical, mechanical environment and 7) promote regenerative therapy to enable shorter healing times. (see **Figure 2-2**)

In this work, it is hypothesized that a modular layer-by-layer (LBL) polymer film formed *in situ* can be designed to meet these goals. Through a series of oral mouth rinses utilizing biotin-streptavidin affinity linkages, a multilayered polymeric system can be developed as illustrated in **Figure 2-3**. The current proposed treatment strategy is expected to provide significantly better barrier function to overcome shortcomings seen from bulky bioadherent film/gel systems. The formulated multilayered barriers are expected to be unaffected under intra-oral environments and remain wear resistant by utilizing strong affinity-based linkages. Such multilayered polymeric systems (LBL), by offering a protective stable barrier effect, enhance the regenerative wound healing response among affected patients. Also, the potential ability of LBL systems to function as a drug carrier vehicle can be used for oral delivery applications.

The objective of this work is to evaluate the hypothesis that multilayered systems can provide a desired long lasting barrier effect with a potential ability to deliver drugs localized to oral mucosal wounds. Yet, in order to realize this, a LBL system must both be chemically and mechanically stable. In this work, LBL barriers are formed and evaluated for their *in vitro* stability in protease and salivary fluids. Further, to demonstrate their ability to decrease surface interaction, *ex vivo* adhesions studies are performed.

Table 2-1: Evaluating severity of oral mucositis - various scoring methods with their employed method of assessment.

Oral Mucositis scoring method	Grades/ Scores/ Stages					References
WHO	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	[28, 75]
	None	Soreness, erythema	Erythema, ulcer can uptake solid foods	Ulcer, can uptake only liquid diet	Impaired/ inability to provide any oral nourishment	
NCI-CTC (for chemotherapy)	None	Erythema, painless ulcers, mild soreness, without lesions	Painful erythema, ulcers but possess ability to eat or swallow	Painful erythema, edema, ulcers, inability to eat thereby requires intravenous hydration	Severe ulceration, require nutritional support or prophylactic intubation	[28, 51]
NCI- CTC (for radiotherapy)	None	Erythema	Patchy pseudo-membranous patches (diameter ≤ 1.5 cm)	Confluent pseudo-membranous patches (diameter >1.5 cm)	Necrosis, deep ulceration, may include bleeding not induced by minor trauma or abrasion	[28, 50, 76]

Table 2-1 (Continued)

NCI- CTC (for bone marrow transplants)	None	Erythema, painless mild soreness, without lesions	Painful erythema, edema, ulcers but with ability to swallow	Painful erythema, edema, ulcers preventing swallowing, and requires hydration or parenteral nutritional support	Severe ulceration requires prophylactic intubation or resulting in documented aspiration pneumonia	[28, 50, 76]
Oral Mucositis Assessment Scale (OMAS)	Scores= 0 to 3 Visual analog scale = 0 to 100, where 100 = worse condition					[51, 77]
	Score= 0 No lesions	Score= 1 Lesions < 1cm ²	Score= 2 Lesions 1-3 cm ²	Score= 3 Lesions >3 cm ²	N/A	
World Consortium for Cancer Nursing Research (WCCNR)	Stages= 0 to 3					[50, 78]
	Stage = 0 Lesions= 0 Normal pink, no edema, no infection	Stage = 1 Lesions = 1 to 4 Slight redness, mild edema, no infections but mild discomfort	Stage = 2 Lesions > 4 (not coalescing) Moderate redness, edema, mild xerostomia, difficulty in eating expect liquid or bland foods	Stage = 3 Coalescing lesions, total denudation of oral cavity, increased redness, severe edema and pain, marked xerostomia, inability to eat or drink	N/A	

Table 2-2: Use of non-therapeutic method as a prophylactic strategy in OM.

Strategy	Generic name	Trademark/ Brand name	Functional properties	Level of success	Mode of action	References
Oral cryotherapy	Cryotherapy	-NA- Cheap, readily available method	Oral cooling results in localized vasoconstriction.	<ul style="list-style-type: none"> • Contributes to a decrease in incidence and severity of OM. • Causes discomfort in patients. 	Cooling effect results in a reduced blood flow, decreasing drug distribution in mucosal tissues thereby greatly reducing OM incidence.	[79-81]
Laser therapy	Low-intensity laser therapy	-NA-	Laser therapy enables neovascularization and brings forth anti-inflammatory response.	<ul style="list-style-type: none"> • Reduces the severity of oral mucositis. • Inhibits inflammatory response. 	<ul style="list-style-type: none"> • Cyclooxygenase-2 (COX-2) inhibition. • Upregulates vascular endothelial growth factor (VEGF) affecting angiogenesis. 	[82, 83]

Table 2-3: Use of topical analgesics as a prophylactic strategy in OM.

Strategy		Generic name	Trademark/ Brand name	Functional properties	Level of success	Mode of action	References
Topical analgesic	Oral mouth rinse	Benzydamine hydrochloride	Tantum™	<ul style="list-style-type: none"> • Benzydamine hydrochloride is a non-steroidal drug. • Produces anti-inflammatory and topical analgesic effects. 	<ul style="list-style-type: none"> • Shows prophylactic effect in radiotherapy (HNC, low doses). • But not effective in chemotherapy induced OM. 	<ul style="list-style-type: none"> • Inhibits inflammatory cytokines production. • Effects in anti-inflammation. 	[81, 84, 85]
	Buccal tablets / Oral spray	Lidocaine spray/tablets (Lidocaine hydrochloride, carbopol, sodium carboxymethyl cellulose, polyvinyl-pyrrolidone, mannitol was used in tablets)	Altaseptic MM / Xylocaine Administration <ul style="list-style-type: none"> • oral spray • buccal tablets through muco-adherence on inner lip 	<ul style="list-style-type: none"> • Licodaine acts as an analgesic similar to benzydamine in function. • Provies local anaesthetic effect. • Anionic polymers acts as a mucoadherent, also sustains drug release. 	<ul style="list-style-type: none"> • Licodaine is effective in pain-relief, mostly when used in HSCT induced OM. • Overdose possess a risk of CNS toxicity (few were dizziness, delirium, muscle twitching etc). 	<ul style="list-style-type: none"> • Provides a localized delivery diffusing from the tablet. • Expected to provide pain relief with its analgesic effects. 	[86, 87]

Table 2-4: Use of various biology response modifiers as a prophylactic strategy in OM.

Strategy	Generic name	Trademark/ Brand name	Functional properties	Level of success	Mode of action	References
Biological response modifier	TGF- β 3 (Transforming growth factor β 3)	Intraepithelial delivery (Drug with Chitosan gel (Carrier))	<ul style="list-style-type: none"> Chitosan functions as a muco-adherent. Gel carrier protects the therapeutic drug preventing rapid release. 	<ul style="list-style-type: none"> Early administration improves wound healing. Ineffective when administration was delayed (after 3 or 5 days during cancer therapies). 	<ul style="list-style-type: none"> TGF-β3 decreases oral epithelial proliferation. Prevents tissue damage from toxic effect of cancer treatments. 	[60, 88, 89]
	KGF1 (keratinocyte growth factor) Also known as fibroblast growth factor (FGF-7)	Palifermin/ Kepivance™ systemic delivery Intravenous administration (first approved preventive agent)	<ul style="list-style-type: none"> KGF helps in wound healing by its ability to mediate reepithelialization. Utilizes mesenchymal-epithelial interactions for tissue healing. 	<ul style="list-style-type: none"> Reduces the need for opioid analgesics in HSCT. Such effects were inconclusive among OM from chemo or radiation therapies. 	<ul style="list-style-type: none"> Downregulates pro-inflammatory cytokines. Stimulates detoxification enzymes. Cytoprotective effect. Possess regenerative effects enhances cellular migration, proliferation and differentiation. 	[60, 90-93]
	EGF (Epidermal growth factor)	-NA-EGF spray providing topical delivery to injured oral mucosa	<ul style="list-style-type: none"> Upregulates growth rate of epithelial cell and fibroblasts. Aids in cell renewal. 	rhEGF (human recombinant EGF) showed therapeutic effect on OM induced from chemo/ radiotherapy.	<ul style="list-style-type: none"> Possess cytoprotective effect. Brings forth homeostasis. Positively affects angiogenesis. 	[94-96]

Table 2-5: Use of various oral mouth rinses as a treatment strategy in OM.

Strategy	Generic name	Brand name	Functional properties	Level of success	Mode of action	Reference
Oral mouth rinse	Supersaturated calcium phosphate mouth rinse. (dibasic/monobasic sodium phosphate, sodium/calcium chloride)	Caphosol® artificial saliva	<ul style="list-style-type: none"> Inorganic phosphate plays a role in regenerating damaged mucosa. Calcium ions provide anti-inflammation, vasodilatation and coagulating effects enabling tissue repair. 	<ul style="list-style-type: none"> Significantly effective in BMT patients. Effective against hyposalivation and xerostomia Enables reduction in pain and disease duration) 	<ul style="list-style-type: none"> Moistens and provides a lubricative effect on oral cavity. Intended to restore pH and normal ionic balance in oral cavity. 	[64, 97-100]
	(No standard formulation) Maalox®, milk of magnesia, kaopectate, antifungals, topical steroids, topic anesthetics such as Benadryl® or licodaine	Magic mouth wash	<ul style="list-style-type: none"> Combination of ingredients such as antibiotic, antihistamine, antifungal, steroid, local anesthetic or antacid. Mixture of atleast above 3 mentioned ingredients. 	<ul style="list-style-type: none"> Ineffective, effects were no different in comparison to ordinary saline washes. Can result in drowsiness and numbness of oral cavity including tongue. 	By utilizing the individual therapeutic effects of formulation, it was expected to meet desired clinical need (pain relief and reduction in healing time).	[4, 69, 93]
	Chlorhexidine gluconate mouthrinse	Oradex®	Chlorhexidine is a dicationic chlorophenyl biguanide, which possess excellent anti-bacterial properties.	Effective in plaque control.	<ul style="list-style-type: none"> Chlorhexidine functions as a bacteriostatic agent (inhibits) at low concentration. Bactericidal (kills) at high concentrations. 	[101, 102]

Table 2-6: Use of Mucoadhesive gels as a treatment strategy in OM.

Strategies	Generic name	Trademark/ Brand name	Functional properties	Level of success	Mode of action	References
Mucoadhesive gels	Polyvinyl pyrrolidone (PVP), hyaluronic acid (HA), glyceric acid (GA)	Gelclair®	<ul style="list-style-type: none"> • PVP, HA are muco-adherents which form protective films. • GA breaks down active component in flavoring agent. 	Short-term pain relief, temporary improvement in ability to eat or drink	<ul style="list-style-type: none"> • Adheres to oral mucosa forming a protective barrier over ulcerous lesions. 	[103-106]
	Acemannan hydrogel™ (polymeric acetylated mannans derived from aloe vera extracts)	RadiaCare™	Hydrogels derived from aloe vera possess growth substance which improves anti-inflammatory and wound healing activity	<ul style="list-style-type: none"> • Not conclusive (conflicting results). • Equivalent to Gelclair® in its mode of action. 	<ul style="list-style-type: none"> • Provides temporary pain relief through tissue hydration and from lubricative effect (Palliative therapy). 	[103, 107-111]
	Xylitol, hyaluronan	Gengigel®	<ul style="list-style-type: none"> • Hyaluronan (HA) is a key component in extracellular matrix. • HA helps in migration, proliferation and differentiation of keratinocytes. • Xylitol functions by slowing down bacterial growth. 	<ul style="list-style-type: none"> • Rapid pain relief, although effects are temporary. • Weak antibacterial effect. • Cannot be recommended for plaque control. 	<ul style="list-style-type: none"> • Apart from barrier effects, hyaluronan in Gengigel® was expected to provide anti-inflammatory effect. • Enables re-epithelization and tissue regeneration. 	[102, 112, 113]



a. Grade 1: erythema of the mucosa



c. Grade 3: confluent ulcerations or pseudomembranes; bleeding with minor trauma



b. Grade 2: patchy ulcerations or pseudomembranes



d. Grade 4: tissue necrosis, significant spontaneous bleeding, life-threatening consequences

Figure 2-1: Different grades of oral mucositis as per NCI assessment scales.

Adapted taken from feature article by Cawley, M.M. and L.M. Benson, Current trends in managing oral mucositis, Clin J Oncol Nurs, 2005. 9(5): p. 584-92. [17]).

[(Photo courtesy: Mark Schubert, MSD), Reproduced with permission]

EFFECTIVE TREATMENT STRATEGIES AND ASSOCIATED TREATMENT BARRIERS

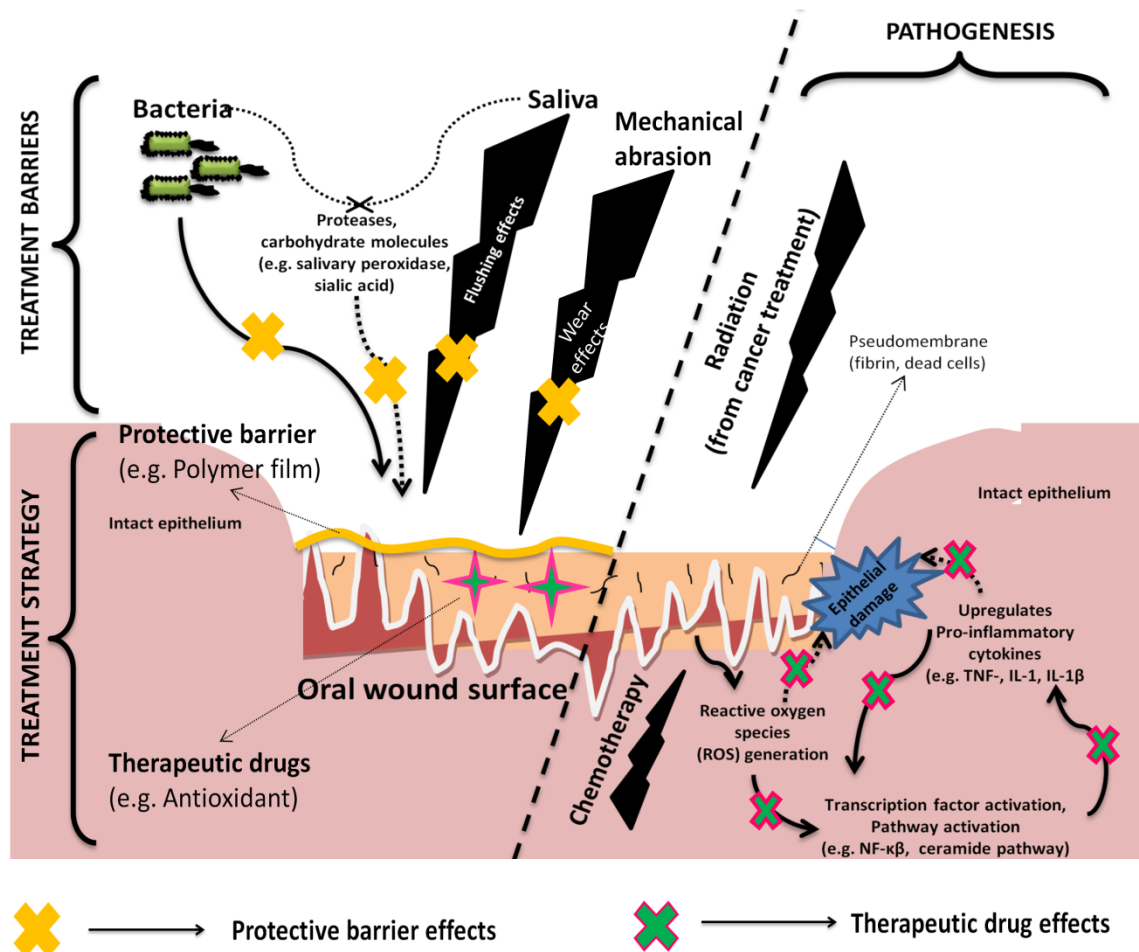
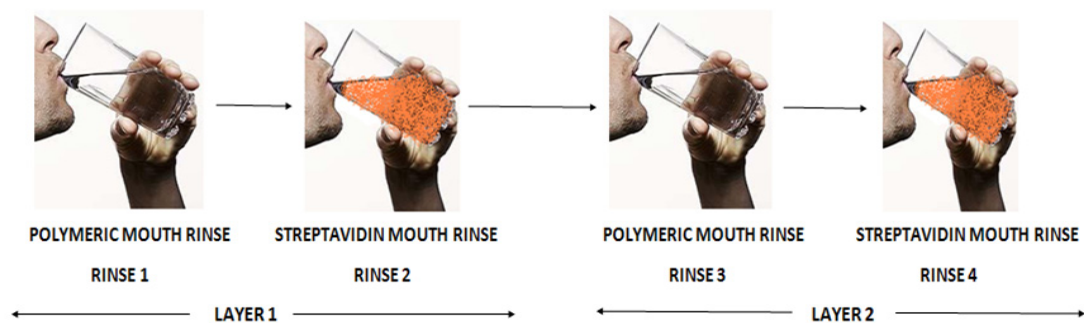


Figure 2-2: Scheme showing pathogenesis of oral mucositis, potential treatment strategies and treatment barriers associated with oral mucosal wounds. Treatment strategies (protective barriers, therapeutic drugs) and its effects on treatment barriers and OM pathogenesis were also shown.



ORAL DRUG DELIVERY SCHEME

Figure not drawn to scale

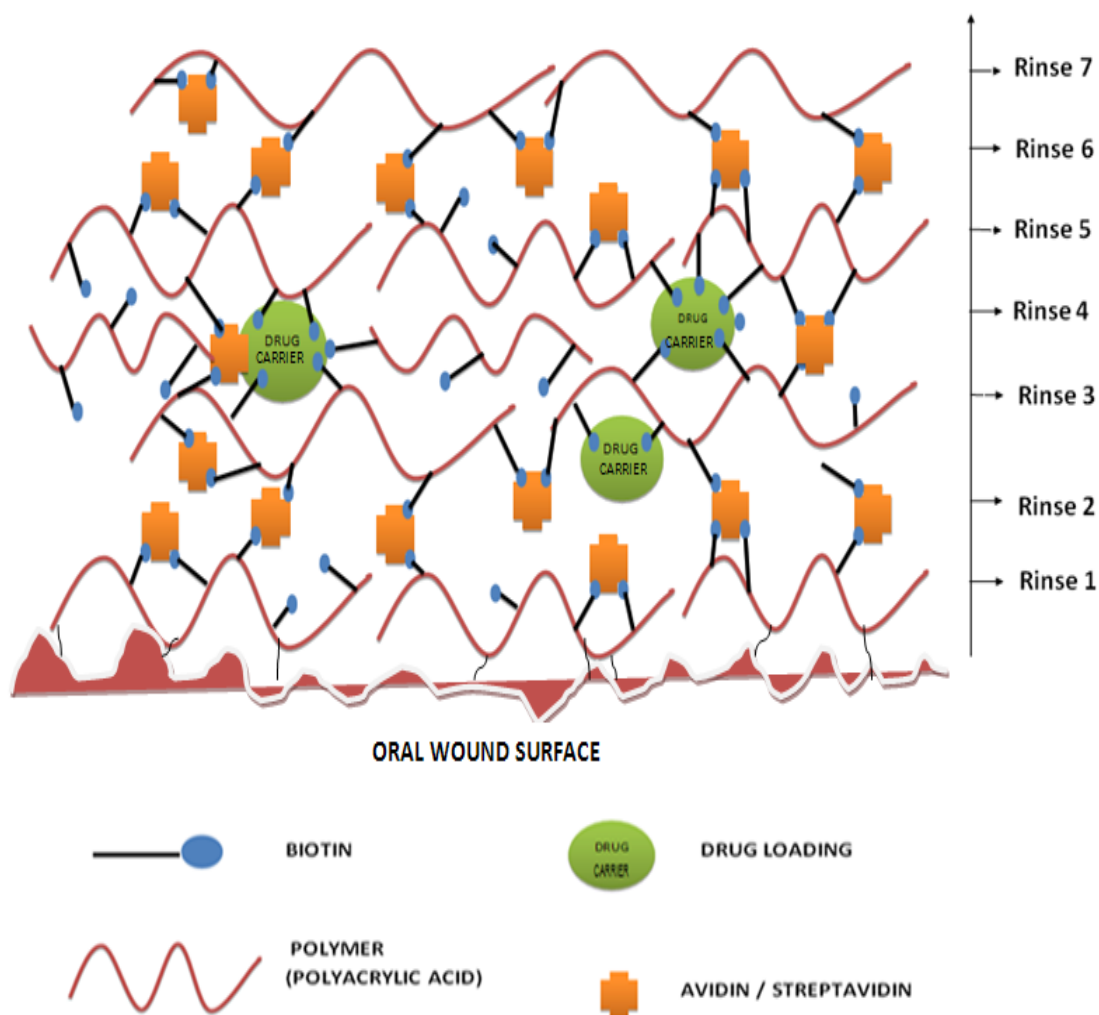


Figure 2-3: Scheme showing overall proposed application of LBL self- assemblies in oral drug delivery.

CHAPTER 3. RESEARCH GOALS

3.1. Objective/Hypothesis

Multilayered affinity based polymeric systems can provide a durable protective barrier over injured oral mucosa, offering a regenerative treatment strategy through their resistive stability against the harsh intraoral environment.

3.1.1. Specific aim #1: Synthesize and characterize biotin functionalized polymers.

1. Synthesize biotinylated poly(acrylic acid) of different polymeric molecular weights and extents of biotin conjugation.
2. Characterize product purity and degree of biotinylation using reverse-phase high performance liquid chromatography (RP-HPLC) and HABA (4'-hydroxyazobenzene-2-carboxylic acid) analysis [114].

3.1.2. Specific aim #2: Formulate LBL self-assemblies and evaluate *in vitro* chemical stability.

1. Develop *in vitro* LBL assemblies from synthesized biotin functionalized polymers and assess extent of self-assembly growth for different polymer molecular properties.
2. Evaluate LBL chemical stability and barrier properties through *in vitro* tests in physiologically relevant oral salivary and protease medium.
3. Estimate key factors that contribute to LBL development and LBL chemical stability through exploratory statistical models and multivariate analysis.

3.1.3. Specific aim #3: Evaluate LBL mechanical stability through *ex vivo* adhesion studies.

- 1) Perform adhesion tests to demonstrate LBLs physical barrier effect in preventing adhesion from surrounding tissues.
- 2) Evaluate LBLs mechanical stability (durability) through repeat contact barrier tests to analyze their contact wear resistance.

CHAPTER 4. POLY(ACRYLIC ACID) BIOTINYLATION AND CHARACTERIZATION

In biochemistry, the process of conjugating biotin to proteins or macromolecules is known as biotinylation. The biotinylated macromolecules in combination with protein avidin / streptavidin has potential for a wide variety of applications utilizing the strong non-covalent affinity between biotin-(strept)avidin linkages. Biotin-streptavidin complexes form due to the combined effect of several hydrogen bonds and van der Waals interactions which lead to a high association coefficient (K_a) in the order 10^{15} M^{-1} [115]. Such high affinity in biotin-streptavidin complexes is nearly 10^3 - 10^6 times greater than antibody-ligand interactions [116]. The use of such characteristic biotin-streptavidin interactions in multilayered self-assembly enables a strong affinity and specificity which are unaltered by environmental pH variation and ionic concentration. Biotin-streptavidin interactions also possess a very high thermal stability [117]. Hence, to develop such stable affinity based multilayered polymeric barriers; biotin functionalization of polymer (biotinylation) represents an indispensable step. In this study, poly(acrylic acid) (PAA), a weak polyanion with known anti-bacterial effects and mucoadhesive properties is used in biotinylation synthesis [118, 119]. The adhesive components in oral pathology (e.g., fibronectin and laminin) contain many positively charged residues (e.g., arginine, lysine) [120, 121]. Hence, the addition of oppositely charged polyanion PAA (negative polymeric backbone), is expected to improve polymer binding capability due to electrostatic interactions.

4.1. Materials and methods

4.1.1. Materials

Poly(acrylic acid sodium salt) (MW 10,000 and 50,000 Da) and PAA (MW 90,000 Da) were purchased from Polysciences, Inc. (Warrington, PA) and were lyophilized prior to use. During lyophilization, PAAs in aqueous solution were frozen by mechanical refrigeration and freeze dried to sublimate water. Polymer PAA was collected as dry powdered mass. All other chemicals were used as purchased without any further purification unless stated. Pentylamine-biotin and avidin were purchased from Thermo Scientific (Rockford, IL). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC.HCl), 2-(N-morpholino)ethanesulfonic acid (MES) and 2-(4-hydroxyphenylazo)-benzoic acid (HABA) were purchased from Sigma Aldrich (St.Louis, MO). N-hydroxysuccinimide (NHS) was purchased from Acros Organics (NJ).

4.1.2. Polymer biotinylation synthesis

PAA biotinylation was carried out with minor modifications to a previously published procedure [122]. In polymeric biotinylation by EDC-NHS coupling chemistry, the molar ratio of PAA: EDC: NHS (1:10:20) was fixed. The extent of biotin conjugation on PAA was altered based on molar ratio of pentylamine-biotin to carboxylic acid repeat unit in PAA. To obtain a low extent of biotin conjugation [Biotin+], a molar ratio of pentylamine-biotin to PAA of 1:5 was used, whereas for a high extent of conjugation [Biotin++] the molar ratio used was 1:2.5. All of the materials were weighed out to meet the desired molar ratios. PAA, EDC and NHS were dissolved in 50 mM MES buffer and transferred into a sealed flask at room temperature with a regulated pH of 5.0-6.0. To

the reaction mixture, pentylamine-biotin was added immediately and the pH was adjusted by either acid (HCl) or base addition (NaOH) to 7.0-8.0, in order to enhance the reaction of carboxylic acid with amine to form a stable amide bond. The reaction was carried out for a period of 24 hrs.

4.1.3. Purification and characterization

The biotinylation reaction mixture was filtered to remove any precipitate suspensions (N-acylurea) formed during reaction. Further purification was carried out using ultrafiltration (Millipore, USA), allowing for the removal of excess unreacted pentylamine-biotin, EDC and NHS from the synthesized biotinylated PAA (Biotin-PAA) product. RP-HPLC (Shimadzu Prominence) was carried out on ultrafiltered biotinylation reaction samples. An isocratic mode with a mobile phase of acetonitrile (organic phase) and water with 1% trifluoroacetic acid (aqueous phase) in (15:85) % volume ratio was used in RP-HPLC analysis. All purified reaction samples were subjected to HABA-avidin assay. HABA stock solution and HABA-avidin working solution was prepared as outlined by Shuvaev et al.[114]. To the HABA-avidin working solution (red/orange colored), purified reaction samples from polymer biotinylation synthesis was added. This addition resulted in color change (pale orange/ yellow) which was measured from the decrease in the absorbance at 500 nm using a UV-Vis spectrophotometer (Cary WinUV). By comparing calibration made from known free biotin addition to HABA-avidin working solution, the actual estimate of extent of biotinylation in different synthesized biotinylated PAA molecules was determined.

4.2. Results and discussion

4.2.1. Polymer biotinylation

PAA was functionalized with biotin through carbodiimide chemistry [123-126]. With an aim of developing LBL polymeric self-assemblies, biotin moieties were incorporated into polymeric PAA chain. For biotin functionalization the most commonly used reagents are N-hydroxysuccinimide (NHS) esters or N-hydroxysulfosuccinimide (Sulfo-NHS) esters for carboxylic acid reaction with primary amines. 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC.HCl) is commonly used as a zero-length aqueous phase cross linker in combination with NHS / Sulfo-NHS esters to form a stable amide bond in biotinylated product. The proposed reaction scheme is shown in **Figure 4-1**. During the biotinylation reaction, an O-acylisourea based intermediate is expected to be formed by EDC activation on carboxylic acid (PAA). In the absence of amino groups (pentylamine-biotin), this intermediate hydrolyzes to form precipitate by-product N-acylurea [127, 128]. These intermediate were filtered off and other excess components were removed from biotinylated product by ultrafiltration.

The presence of free unreacted pentylamine-biotin in reaction sample would compete with biotinylated polymer product and thereby limits the use of 2-(4-hydroxyphenylazo) benzoic acid (HABA) analysis in determining the extent of PAA biotinylation. Hence reaction sample purification was carried out for an extended duration until free pentylamine-biotin was completely removed (below negligible limits). To detect the degree of the free pentylamine-biotin during and after reaction sample purification, a reverse phase high performance liquid chromatography (RP-HPLC)

analysis was carried out. From RP-HPLC analysis on all purified samples, complete removal of free pentylamine-biotin (peak disappearance) was ensured; thereby its product purity (> 99%) (see **Figure 4-2**). All post-purified reaction samples were subjected to HABA analysis by addition to HABA-avidin complex. This addition replaces the weakly bound HABA molecules ($k_d = 10^{-6}$ M) from HABA-avidin complex by a higher affinity biotin molecules from biotinylated PAA ($k_d = 10^{-15}$ M) [129, 130], described in scheme **Figure 4-3**. The release of free HABA results in a color change which was quantified for synthesized reaction samples by the decrease in its absorbance at 500 nm (see **Figure 4-4**). The extent of biotin conjugation to PAA chains was determined for synthesized biotinylated PAAs of different polymer molecular weight (**Figure 4-5**). As expected, biotinylated PAAs within same polymer MW (10,000/50,000/90,000) which were synthesized from higher pentylamine-biotin to PAA molar ratio ([Biotin++]= 1:2.5) contained an increased number of biotin molecules incorporated in polymer chain as opposed to products prepared with a lower molar ratio ([Biotin+]= 1:5). Among conjugated polymers, "[Biotin++]/PAA 90" possess the highest number of biotin molecules per PAA chain (7.14 biotin molecules), whereas "[Biotin+]/PAA 50" and "[Biotin+]/PAA 10" contained the least number of biotin molecules per PAA chain (1.2 biotin molecules).

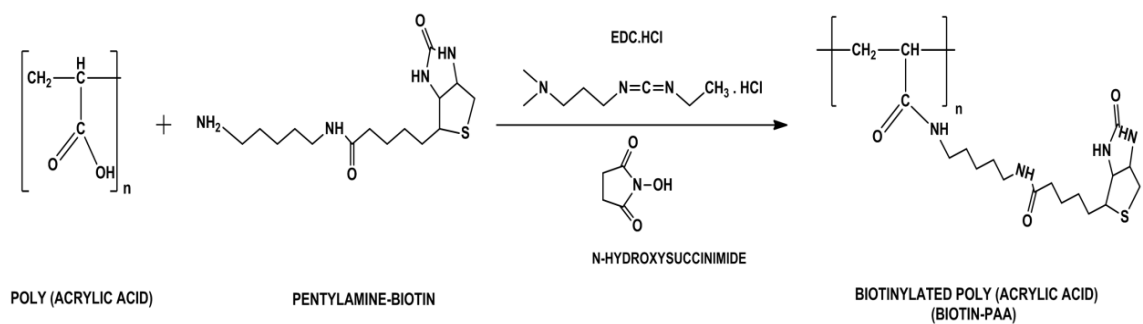


Figure 4-1: Synthesis scheme for poly(acrylic acid) biotinylation. Plot shows EDC-NHS coupling chemistry by reaction of carboxylic acid from PAA and amine group from pentylamine-biotin to form a stable amide bond in product biotinylated PAA (Biotin-PAA).

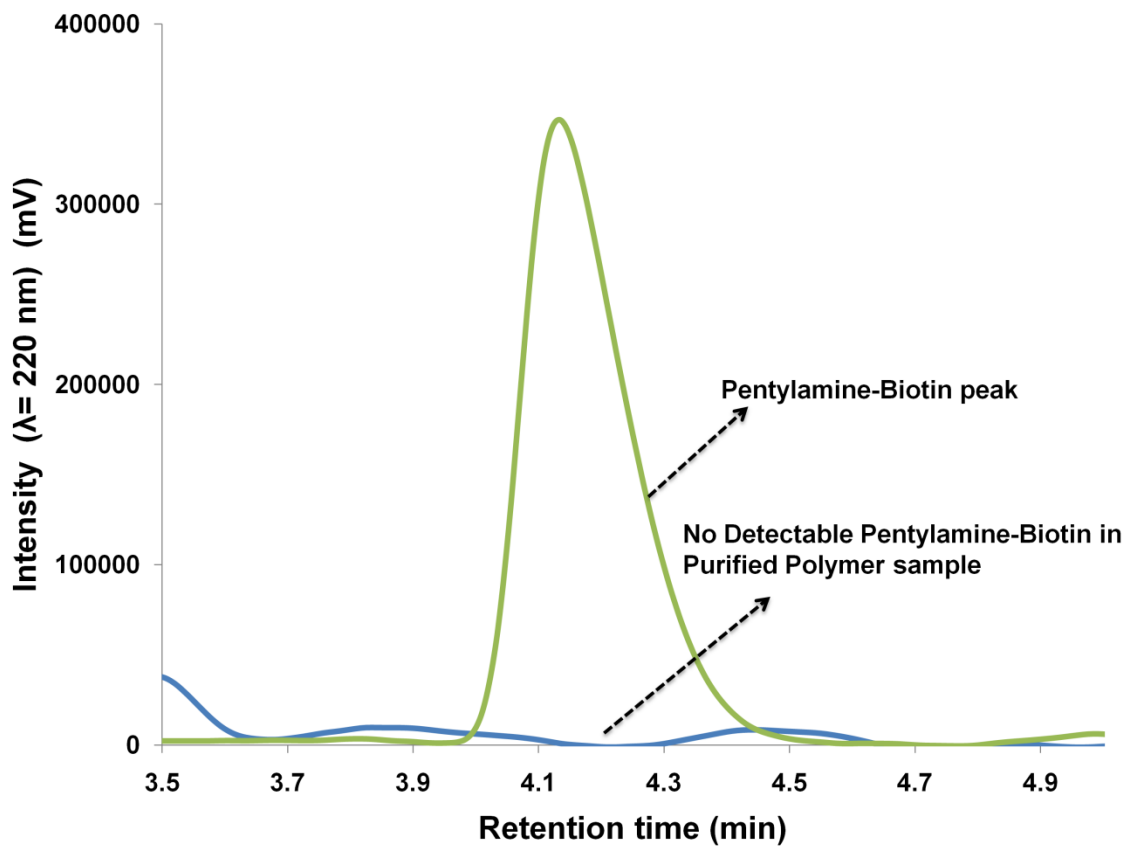


Figure 4-2: Reverse phase HPLC for detecting removal of unreacted biotin from reaction sample. Plot shows free pentylamine-biotin peak (reference peak) and disappearance of unreacted pentylamine-biotin on purification of biotinylation reaction samples (polymer-biotin concentration ~0.12 mM).

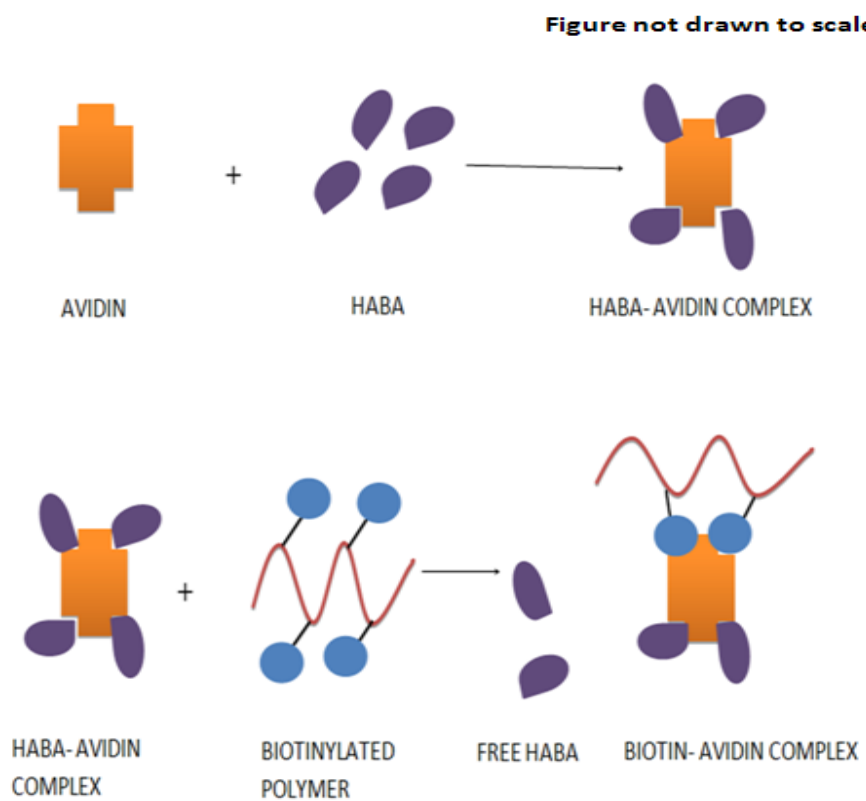


Figure 4-3: Scheme showing mechanism of HABA-avidin analysis which was used to determine extent of biotinylation on ultrapurified reaction samples.

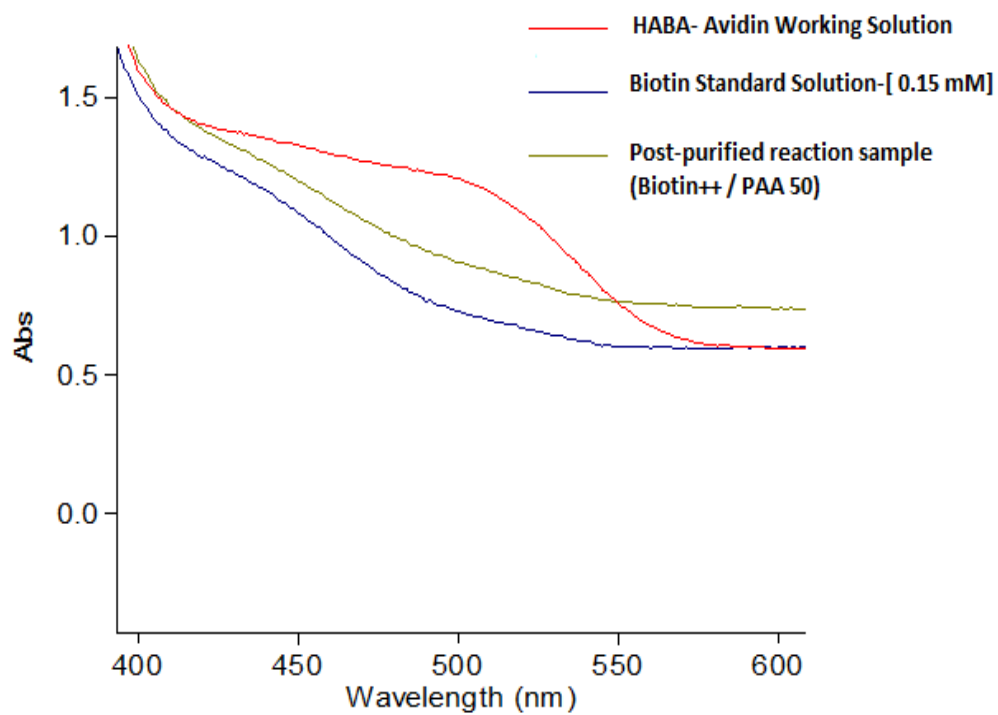


Figure 4-4: UV-visible spectra of HABA-based assays for biotin. Extent of biotinylation is determined through free HABA release where color change was detected by a decrease in absorbance at 500 nm.

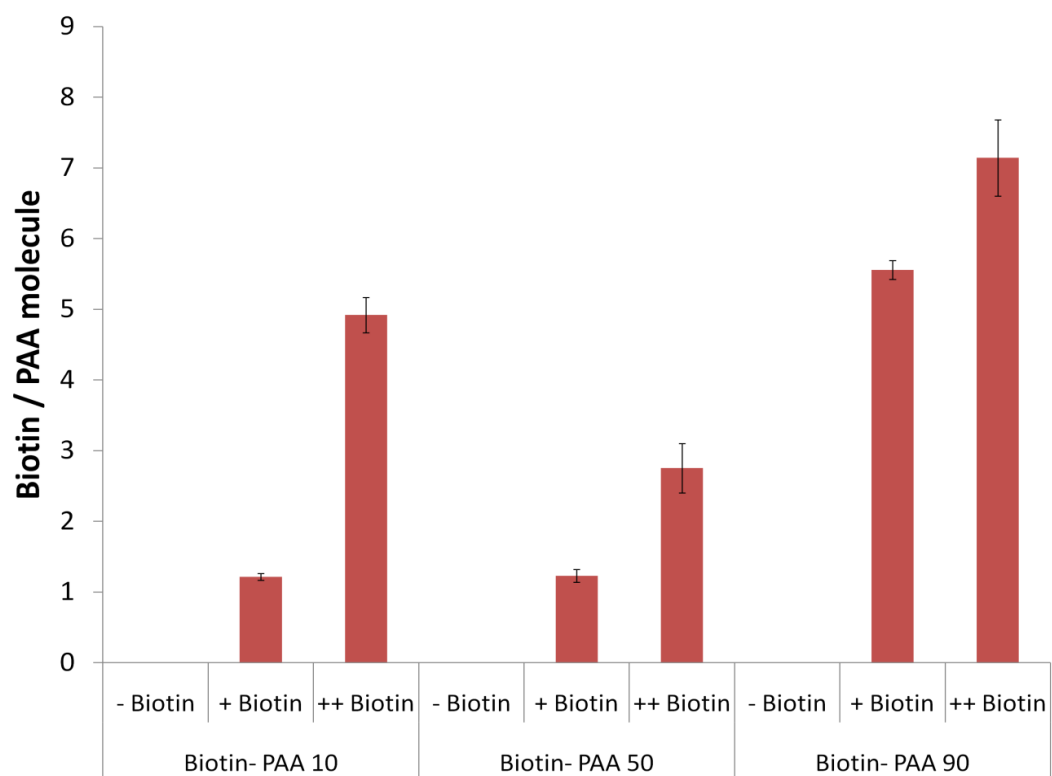


Figure 4-5: HABA analysis results showing extent of polymer biotinylation.

CHAPTER 5. CHEMICAL STABILITY TESTS ON LAYER-BY-LAYER SELF-ASSEMBLIES

The oral cavity is a complex environment containing bacteria, surfactants, carbohydrate molecules (e.g. sialic acid) and wide variety of proteins or enzymes (e.g. salivary peroxidase, lysozyme, α -amylase, carbonic anhydrase, fibronectin, secretory Immunoglobulin A (sIgA), matrix metalloproteinases, kallikrein and lactoferrin) [131]. It is possible that the effect of such diverse components could destabilize the LBL assemblies yielding to proteolytic or salivary surfactant effects. Hence in evaluating such possibility of premature barrier failure, an *in vitro* chemical stability study is performed on LBL systems.

A bacterial protease enzyme “pronase” isolated from *Streptomyces griseus* is used for studying LBL proteolytic stability. Pronase compositions typically contain different exopeptidases and endopeptidases, which explains its non-specific proteolytic activity[132]. An example of one such endopeptidase enzyme is serine protease, which can hydrolytically break down peptide bonds on the carboxyl part of aspartic or glutamic acid yielding non-specific protease behavior[133]. To evaluate the effects of oral saliva on LBL assemblies, an unstimulated whole saliva (UWS) is used. Although salivary medium possesses various components such as water, sugar, carbohydrates, inorganic molecules, proteins and lipids, the effects of its rheological properties are mainly derived from its salivary mucins. Mucins are glycoproteins which can influence salivary viscosity and its spinnability (spinnbarkeit). UWS contains higher mucin level as opposed stimulated whole saliva (generated during chewing) [134]. Hence, LBL barriers were

studied under UWS to evaluate their possible de-assembling tendency in surfactant like properties of UWS.

5.1. Materials and methods

5.1.1. Developing affinity based Layer by Layer (LBL) assemblies

Radiolabeling protein streptavidin: Protein streptavidin was radiolabeled (^{125}I -Streptavidin) and was used in studying LBL self-assembly development using a radiotracing method. Iodogen[®] iodination reagent (1,3,4,6-tetrachloro-3 α -6 α -diphenylglycouril) (Thermo Scientific, Rockford, IL) was dissolved in an organic solvent chloroform and was added to a glass tube (2 mg/ml, 200 μl). The volatile chloroform solvent was evaporated off from iodogen[®] reagent using dry nitrogen gas to form a thin film covering the glass tube. Cold streptavidin (100 μl of 1mg/ml solution) was introduced into the iodogen coated test tube, to which radioactive iodine (^{125}I) was added (~30 μCi) and incubated for at least 5 minutes to enable iodination of tyrosyl groups of streptavidin [135]. The free iodine was removed from radiolabeled streptavidin by spinning down in gel filtration columns (Biospin[®] 6 Tris columns).

Free iodine determination: The extent of free iodine content in the spin down product (radiolabeled streptavidin) was estimated by the trichloroacetic acid (TCA) precipitation method. TCA (20% (w/v), 1 ml) was added to protein mixture containing radiolabeled streptavidin (2 μl of 1 mg/ml radiolabeled protein stock) and bovine serum albumin (BSA) (1ml of 5% (w/v)). The TCA addition readily precipitates the protein mixture (incubated for atleast 10 minutes) where the BSA presence aids in forming an appreciable protein precipitate mass to separate radiolabeled proteins from free iodine

by centrifugation. Pellets collected after centrifugation contained the precipitate from radiolabeled streptavidin and cold BSA, whereas the supernatant solution was rich in free iodine. By measuring radioactive gamma counts on supernatants and precipitates separately, the extent of free iodine present was determined. All batches of radiolabeled streptavidin used in the studies had minimal free iodine content (< 10%).

5.1.1.1. Base layer adsorption studies

To establish an initial base layer by which LBL can be formed, streptavidin adsorption to radioimmunoassay (RIA) plates was performed. 100 µl of ¹²⁵I-streptavidin (1- 1000 nM) was added to the plates and was incubated for 1 minute, and then washed at least 3 times with phosphate buffered saline (PBS). The amount of protein adsorbed was measured using a gamma counter (Perkin Elmer).

5.1.1.2. *In vitro* LBL

In vitro LBL studies were carried out on RIA plates. Base streptavidin (Layer 1) was formed on the RIA microplates on the basis of the base layer adsorption study data. The vacant adsorption sites in the base layer were blocked using 5 wt% bovine serum albumin (BSA) by providing an incubation time of at least 2 minutes. The unbound BSA was removed by a series of PBS washes (at least 3 rinses). To the base streptavidin layer, an alternating rinse of biotinylated PAA (2 minute incubation, 100 µl) and streptavidin rinse (1 minute incubation, 100 µl) were provided. The molar ratio of biotinylated polymer to streptavidin ratio was kept atleast 4 fold in excess to ensure all streptavidin binding sites were occupied during LBL formation. The excess biotinylated polymer or

streptavidin was removed by intermittent PBS rinses (atleast 3 times, 200 μ l). To study the LBL development, self-assemblies with different numbers of streptavidin layer in the LBL system (NoL = 1, 3, 5, and 7) were characterized for various synthesized biotinylated PAA materials.

5.1.2. Chemical stability tests on *in vitro* LBL assemblies

Chemical stability tests were carried out on the developed *in vitro* LBL systems. Either unstimulated whole saliva (UWS) or protease enzyme pronase were used to study LBL chemical stability. UWS was collected by the spitting method, with a harvesting lag time of at least 2 hours from meal intake. Other variable salivary factors were kept minimal by abstinence from smoking or drinking [136]. For proteolytic stability test, pronase medium (0.01 % w/v) was used. Developed *in vitro* LBL systems were incubated in oral salivary medium or in pronase medium for different times. Streptavidin mass remaining in the LBL system after various incubation times was measured using gamma counter.

5.1.3. Statistical analysis

Principal component regression (PCR) was performed using statistical software (The Unscrambler® V9.7 and V10.1, Camo Software Inc) on the results of *in vitro* LBL development and LBL chemical stability tests. The response variable, fractional increase in radiolabeled streptavidin mass from the base layer " M_L/M_1 " derived from *in vitro* LBL tests was used in studying effects of LBL factors (MW, conjugation and NoL).

Similarly response variables ($t_{1/2}$ and mass loss at $t_{1/2}$) derived from LBL chemical stability analysis was used in studying effects of LBL factors (MW, conjugation and NoL). Here, $t_{1/2}$ is the time required for the LBL assemblies to degrade into halve their maximum possible degradation ($1/2 \cdot M_{\infty}$). In PCR analysis, test of significance was performed on regression coefficient 'B' (principal component slope), using uncertainty tests.

Also, main effects of the factors affecting LBL chemical stability were studied using various 3-way ANOVA models (Generalized Linear model (GLM) and 3-way nested ANOVA) [137]. In analyzing *in vitro* LBL development a GLM model was used. Further post-hoc analysis (multiple comparison methods) was performed using Bonferroni, Sidak and Dunnett methods in the GLM model [138]. ANOVA plots containing main effects and interactions plots were obtained for all response variables obtained from *in vitro* LBL development and for LBL chemical stability studies. All 3-way ANOVA models and post-hoc analysis were performed using statistical software (Minitab 16) [139]. For all multivariate analysis (PCR), ANOVA tests and hypothesis testing performed, the effects were considered significant only if $P < 0.05$.

5.1.4. LBL degradation mechanism

To investigate the degradation mechanism, LBL systems with the same number of self-assembly layers (NoL=5) were developed, using a modified *in vitro* LBL procedure. For base layer degradation (Layer 1), the initial protein layer was applied using ^{125}I -streptavidin with all subsequent layers using cold (non-radioactive) streptavidin. A

similar procedure was adopted for studying degradation from the middle layer (Layer 3) and outermost layer (Layer 5) by radiolabeling the specific individual protein layer. Protein mass remaining in individual layers was measured for various salivary (UWS) incubation times using a gamma counter.

5.2. Results and Discussion

5.2.1. Developing Layer-by-Layer self-assemblies

5.2.1.1. *In vitro* LBL assembly formation

For developing multilayered polymeric barriers, high affinity biotin-streptavidin chemistry was used. PAA of different molecular weights (MW 10,000; 50,000; or 90,000 Da) with varying extent of biotin conjugation (Biotin+, Biotin++) were used for LBL formation. A complete set of polymers used is listed in **Figure 4-5**.

In developing *in vitro* LBL assemblies on radioimmunoassay (RIA) plates an initial base layer (Layer-1) of streptavidin was formed (see **Figure 5-1**). With higher concentration of streptavidin addition during initial base layer formation studies, there is a formation of a “high-density layer”, this is likely from a change in protein conformation from side on position to end-on position. Such protein conformational change on the surface could likely induce protein uncoiling or unfolding effects causing denaturation or change in streptavidin active sites (binding pockets) for biotin binding[140]. Hence, for *in vitro* LBL studies, protein coverage in the base layer was fixed below monolayer saturation (189 nM), ensuring developed assemblies were from a single base layer adsorbed to hydrophobic interface [141]. During LBL assembly,

unoccupied sites in base layer were blocked using bovine serum albumin (BSA), minimizing non-specific adsorption effects [142-144]. LBL systems with desired number of streptavidin layers were developed over this protein base layer according to the scheme shown in **Figure 5-2**.

The extent of LBL development was analyzed from the fractional increase in radiolabeled streptavidin mass from the base layer, " M_L / M_1 ". The results show that significantly more material was deposited during LBL assembly growth using high MW biotinylated polymers (Biotin-5.55/PAA 90, Biotin-7.14/PAA 90) (see **Figure 5-3**). The 7-layered assemblies prepared using biotinylated polymers (Biotin-PAA 90) possessed nearly 2.6 fold (161-197 %) higher streptavidin mass than the unconjugated polymer (Biotin-0/PAA90) assemblies, indicating the successful formation of a multilayered assembly as a result of the affinity linkages. Interestingly, the high MW unconjugated polymer (Biotin-0/PAA 90) could form an additional protein layer with its weak charge based interactions, yet were unable to form multilayer structures. Low MW polymers (PAA 10), irrespective of their extent of biotinylation, were unable to form LBL assemblies (**Figure 5-5**). This poor self-assembly formation was due to the polymer's tendency to destabilize the adsorbed protein base layer, most likely due to PAA's surfactant like effects [145] (**Figure 5-5**). LBLs developed using mid-range MW (50,000 Da) biotinylated polymers (Biotin-1.23/PAA 50, Biotin-2.75/PAA 50) possessed moderate LBL assembly formation tendency, as expected (**Figure 5-4**). Self-assembled layers of Biotin-PAA 50 materials were not as substantial as those formed with high MW

polymers (PAA 90), but were better than low MW polymers (PAA 10). This study of molecular weight shows that higher MW enhances the formation of LBL assemblies.

Traditional LBLs formed from polyelectrolyte multilayers (PEM) exhibit exponential (non-linear) layer growth whether the layers are formed using only polypeptides or with biological components [146-148]. Hence, it was hypothesized that the weak non-linear increase in biotin content found in unconjugated high MW polymers (Biotin-0/PAA 90) was likely a result of weak polyanion (PAA) interactions with the protein (streptavidin). PEMs of highly charged polymers typically result in linear growth[146]; it is likely that the nearly linear assembly trend found in LBLs of high MW polyanion PAA (Biotin-5.55/PAA 90 and Biotin-7.14/PAA 90) is due to the significant contributions from stable affinity based biotin-streptavidin linkages.

5.2.1.2. Multivariate analysis on *in vitro* LBL assemblies

Principal component regression (PCR) on *in vitro* LBL was performed to analyze its possible multifactorial dependence and thereby ascertain significant LBL factors that contribute to self-assembled layer growth. The possible LBL factors that influence response variable (M_L/M_1) were polymer MW, extent of biotin conjugation and number of layers (NoL). The results of PCR analysis demonstrated significant main effects from all LBL factors with its interactions (see **Table 5-1**). The significant main effect substantiates that all LBL factors contribute to self-assembly growth by affecting response variable. From interactions, a significant resultant positive influence caused by interdependence of various LBL factors in affecting assembly growth was observed. The

contribution of interactions was shown through various significant 2-way interactions (MW*Conj, Conj*NoL and MW*NoL) and squared interactions (Conj*Conj and MW*MW). This demonstrates the effect of LBL assembly growth was a result of contribution from all LBL factors with its combined main effects and a synergistic role of factor interactions. The results of PCR analysis was compared with analysis from generalized linear model (3-way ANOVA) for its main effects. The main effects were found to be consistent in both the analysis thereby verifies the significant main effect contributions from various LBL factors, shown in **Table 5-2**.

ANOVA plots studying main effect trends in LBL factors showed better assembly formation from increasing polymeric MW (**Figure 5-6**). The main effects showing higher protein mass in assemblies with increasing layers (NoL) reinstates the assembly growth observed from earlier experimental results. The effect of conjugation in distinguishing from unconjugated materials (Biotin-0) was evident at relatively higher biotin conjugations (Biotin- 5.55/7.14). LBL factor interactions, which were found significant through earlier conducted multivariate analysis (PCR model), were analyzed for their trends using two way interactions plot in ANOVA (**Figure 5-7**). A more substantial interaction was evident in systems of LBLs containing higher polymeric MW, better conjugation and with increased assembly layers. Thus, greater interdependence between various LBL factors contributed to enhanced assembly growth.

5.2.2. LBL chemical stability

LBL systems developed for various synthesized Biotin-PAA materials (of different MW/conjugation/NoL) were subjected to *in vitro* chemical stability tests. With an aim of studying intra oral chemical effects, LBL assemblies were subjected to stability under bacterial protease and unstimulated whole saliva (UWS). The effect of bacterial protease (pronase) is likely to offer more significant impact on LBL destabilization with its strong proteolytic effects. Such proteases, however, may exhibit minimal surfactant like effects. In contrast, UWS is likely to possess weak proteolytic effect although its surfactant like properties could dominate de-assembling of LBL barriers.

5.2.2.1. LBL protease stability

In LBL stability under proteolytic pronase, the unconjugated polymers (Biotin-0/PAA 90, Biotin-0/PAA 10) were readily degraded, resulting in poor chemical stability (**Figure 5-8 and 5-11**). The stability of Biotin-0/PAA 90 material was found to be indistinguishable from the streptavidin base layer (Layer 1) further demonstrating the weak association offered by unconjugated polymers (see **Appendix figure A.4.1.**). In biotin conjugated polymers (Biotin-5.55/PAA 90 and Biotin-7.14/PAA 90), aided by their affinity-based linkages, improved LBL stability was noticed (**Figure 5-8**). In studying layer effects, LBLs (Biotin-5.55/PAA 90, Biotin-7.14/PAA 90) with increased number of layers (NoL) yielded better assembly stability (**Figure 5-9**). This improved stability from higher number of assembled layers was a result of better barrier effects from its multilayered polymeric structures. In LBLs of high MW polymer (PAA 90) distinctively better barrier stability was observed as when compared to lower MW polymer (PAA 10), thereby

emphasizing the important role played by polymer MW in affecting LBL stability (**Figure 5-10**). Stability of low MW polymers (Biotin-1.21/PAA 10, Biotin-4.91/PAA 10) was comparable to the streptavidin base layer (Layer 1). This weak barrier stability from PAA 10 materials is likely a result of their shorter polymeric chains which contributed to poor LBL formation and enhanced surfactant like effects, resulting in an enhanced streptavidin detachment from LBL assemblies (**Figure 5-11**). Also, protease stability studies in LBLs of midrange MW (50,000 Da) were performed, and stability results of LBL factor effects are listed in **Appendix section A-5** and **A-6**.

5.2.2.2. Multivariate analysis on LBL protease stability

Multivariate analysis (PCR) was performed on protease stability response using “mass loss at $t_{1/2}$ ” as an indicator of extent of LBL degradation. It was observed that all LBL factors (MW, conjugation and NoL) showed significant contributing effects on degradation rate. Interestingly, with absence of any factor interactions all the effects resulted from direct influence of each factor on extent of LBL degradation, emphasizing an additive/non-synergistic dependence on stability (see **Table 5-3**). In studying factors controlling rate of LBL degradation ($t_{1/2}$), an important role played by polymer MW and number of assembly layers (NoL) was observed with their significant main effect contributions. The role of conjugation and effects of factors interdependence (Interactions) were found insignificant to affect LBL degradation rate, thereby demonstrates its weak influence.

5.2.2.3. LBL salivary stability

Unstimulated whole saliva (UWS) with little to no protease was not expected to significantly degrade streptavidin layers from assemblies. Indeed, detached protein (19-30%) observed in LBLs of PAA 90 materials were likely from surfactant effects of saliva.

In LBLs under UWS, biotin conjugation did not seem to be a strong effecter on barrier stability (**Figure 5-12**). However, layer effects were significant where by increasing LBL layers the protein loss from assemblies was suppressed (**Figure 5-13**). This layer effect was important as with increased network density from increased layered assemblies is expected to form resilient barrier against surfactant qualities of saliva. Polymer molecular weight effects in UWS were similar to protease studies where higher molecular polymers resulted in better stability substantiating the role of polymer MW in LBL stability (**Figure 5-14**). Interestingly, PAA 10 materials resulted in enhanced protein loss from assemblies as observed in protease studies, reasserting earlier described inherent surfactant effects of low MW PAA (**Figure 5-15**).

5.2.2.4. Multivariate analysis on LBL salivary stability

Principal component regression (PCR) on salivary stability studying important factor effects of LBL responses (mass loss at $t_{1/2}$ and $t_{1/2}$) demonstrated significant contributions from main effects of polymeric MW and number of assembly layers (NoL) (**Table 5-4**). Unsurprisingly, the main effects of conjugation were found to be insignificant for all LBL response variables. This substantiates the earlier observed weak

conjugation effects. With absence of interactions for all LBL factors, the absence of factor interdependence was reinstated as was suggested for LBL protease stability.

5.2.3. Statistical analysis on LBL chemical stability

The significance of LBL factors (main effects) with chemical stability response variables were determined using 3-way ANOVA models (see **Figure 5-16**) and compared with earlier conducted PCR analysis. With absence of factor interactions as determined in PCR analysis, the effects of LBL factors on stability response were captured by the factor main effects. The results of PCR analysis were in agreement with the analysis carried out using 3-way ANOVA model (3-way nested ANOVA) thereby confirming the significant effect of MW and NoL on LBL chemical barrier stability, shown in **Table 5-5**. From post-hoc analysis (Bonferroni, Sidak and Dunnet methods) conducted on LBL factors in generalized linear model (GLM), LBL assemblies from biotinylated PAA's of higher MW (90,000 Da) and high NoL (7), consistently resulted in a stable chemical barriers (see **Table 5-6**).

5.2.4. LBL degradation mechanism

LBL degradation mechanism under oral chemical effects can occur by either top-to-down delamination or fissure mode or base layer delamination (see **Figure 5-17**). In top-to-down delamination mechanism, the outer layer of LBLs will be more prone to chemical effect, followed by gradual delamination from outer to inner layers. In this mode, innermost layers are expected to be least susceptible. Under fissure mode, the kinetic rate loss from inner, middle or outer layers in LBLs should be indistinguishable

(uniform) resulting in a porous LBL network on chemical degradation. Other possible degradation mode involves base layer delamination, which through its susceptible inner layer under oral chemical effects can destabilize the LBL assemblies resulting in a sudden rapid loss.

In determining the mechanism through which LBL degradation occurs, 5-Layered assemblies was developed using high MW polymers (PAA 90). LBLs with individual radiolabeled streptavidin layers (innermost or middle or outermost), scheme shown in **Figure 5-18**, were subjected to salivary degradation.

Each individual layer in LBLs of biotinylated polymer (Biotin-7.1/PAA 90) performed better than its respective layer from unconjugated polymer (Biotin-0/PAA - 90) (**Figure 5-19**). This notable increase in stability reinforces the role of multi-layered structures in providing chemical barrier effects as described earlier. The rapid innermost layer loss from unconjugated materials verifies their inability to develop LBL assemblies.

The assemblies of biotinylated polymers followed a top to bottom progression mechanism in chemical degradation suggesting a surface erosion mechanism and an operative polymeric barrier effects from multilayered structures. In LBL systems of biotinylated PAA material (Biotin- 7.1/PAA 90), the outermost layer (Layer 5) was found to be most susceptible to proteolytic degradation. A higher protein loss from outer layer was due to the direct salivary effects in absence of any capping polymeric layer to provide barrier effect. The innermost layer (Layer 1/Base layer) with its overlaid polymeric layers provides an effective barrier effect indicated by its reduced protein loss

from assemblies (**Figure 5-19**). As expected, the middle layer (Layer 3) possessed better stability when compared to outer layer, but resulted in more protein loss in comparison to innermost layers. This barrier tendency exhibited by slow degradation effects of middle and innermost layer was absent in unconjugated materials (Biotin– 0/PAA 90).

Table 5-1: Table shows summarized results of PCR analysis on *in vitro* LBL assembly development, where interactions (2-way and squared interactions) were found to be significant. All LBL factors like MW, conjugation and NoL showed significant main effects for response variable (M_L/M_1). Table contains ‘P’ values obtained from test of significance on regression coefficient (B) where “✓” represents statistically significant factors. Specific factors were considered significant only if $P < 0.05$. (see **Appendix Figure A-2**, for output results obtained during analysis)

p-values for Beta Coefficient	LBL factor effects								
Response variable (LBL growth)	Main effects			2-way Interactions			Squared Interactions		
	M W	Conj	NoL	MW* Conj	MW* NoL	Conj* NoL	MW* MW	Conj* Conj	NoL* NoL
	✓	✓	✓	✓	✓	✓	✓	✓	
M_L/M_1	0	0	0	0	0.0001	0.0001	0.026	0.0001	0.0001

Table 5-2: Summarized results from 3-way ANOVA model (Generalized Linear Model) on main effects of *in vitro* LBL assembly development. Table contains ‘P’ values obtained from test of significance on regression coefficient (B) where “✓” represents statistically significant factors. Specific factors were considered significant only if $P < 0.05$.

Response variable	LBL factors – Main effects		
	MW	Conjugation	NoL
M_L/M_1	✓	✓	✓
	0	0	0.001

Table 5-3: Summarized results of principal component regression (PCR) analysis on LBL protease stability response variables (mass loss at $t_{1/2}$ and $t_{1/2}$) to study effects of various LBL factors (MW, conjugation and NoL). Results were obtained by conducting test of significance (uncertainty tests) on regression coefficient (Slope “B”) derived from principal component analysis. From results, interactions were found to be insignificant whereas factors like MW, NoL showed significant main effects for LBL response variables. Also role of conjugation in affecting mass loss from assemblies were found significant. Table contains ‘P’ values obtained from test of significance on regression coefficient (B) where “✓” represents statistically significant factors. Specific factors were considered significant only if $P < 0.05$. (see **Appendix figure A-7**, for output results obtained during analysis).

p-values for Beta Coefficients	LBL factor effects							
Response variables (LBL protease stability)	Main effects			2-way Interactions			Squared Interactions	
	MW	Conj	NoL	MW* Conj	MW* NoL	Conj* NoL	Conj* Conj	NoL* NoL
Mass loss at $t_{1/2}$	✓	✓	✓					
	0	0	0.0174	0.3063	1	1	0.9775	1
$t_{1/2}$	✓		✓					
	0	0.5778	0.0546	0.8771	1	1	0.9997	1

Table 5-4: Summarized results of principal component regression (PCR) analysis on LBL salivary stability response variables (mass loss at $t_{1/2}$ and $t_{1/2}$) to study effects of various LBL factors (MW, conjugation and NoL). Results were obtained by conducting test of significance (uncertainty tests) on regression coefficient (Slope “B”) derived from principal component analysis. From results, interactions were found to be insignificant whereas factors like MW, NoL showed significant main effects for LBL response variables. Table contains ‘P’ values obtained from test of significance on regression coefficient (B) where “✓” represents statistically significant factors. Specific factors were considered significant only if $P < 0.05$. (see **Appendix figure A-10**, for output results obtained during analysis)

p-values for Beta Coefficients	LBL factor effects							
Response variables (LBL salivary stability)	Main effects			2-way Interactions			Squared Interactions	
	MW	Conj	NoL	MW* Conj	MW* NoL	Conj* NoL	Conj* Conj	NoL* NoL
Mass loss at $t_{1/2}$	✓		✓					
	0	0.1392	0.0092	0.7611	1	1	0.9993	1
$t_{1/2}$	✓		✓					
	0	0.4303	0.0083	0.8392	1	1	0.9996	1

Table 5-5: Summarized results from 3-way nested ANOVA model on LBL chemical stability. Specific factors were considered significant for $P < 0.05$. Design of nested ANOVA model was illustrated in **Figure 5-16**.

Proteolytic medium	Response variable	LBL factors		
		MW	Conjugation	NoL
Pronase	Mass loss % for $t_{1/2}$		✓	✓
		0.122	0.001	0
	$t_{1/2}$	✓		✓
		0.001	0.581	0.006
Saliva	Mass loss % for $t_{1/2}$	✓		✓
		0.005	0.133	0.001
	$t_{1/2}$	✓		✓
		0	0.949	0.011

Table 5-6: Summary of results from 3-way ANOVA models and from post-hoc analysis. Table shows specific significant factors contributing to LBL chemical stability. Specific factors were considered significant for $P < 0.05$. A 3-way ANOVA (Generalized linear model (GLM)) was performed and on performing post-hoc tests (using Bonferroni, Sidak and Dunnett), the following results were obtained.

Response	Proteolytic Environment	Factors								
		Molecular weight		Conjugation				No. of Layers		
		10,000	90,000	1.213	4.919	5.555	7.142	3	5	7
Mass loss @ $t_{1/2}$	Pronase		✓		✓	✓	✓		✓	✓
	Saliva	✓	✓	✓			✓			✓
$t_{1/2}$	Pronase		✓			✓	✓		✓	✓
	Saliva	✓	✓						✓	✓

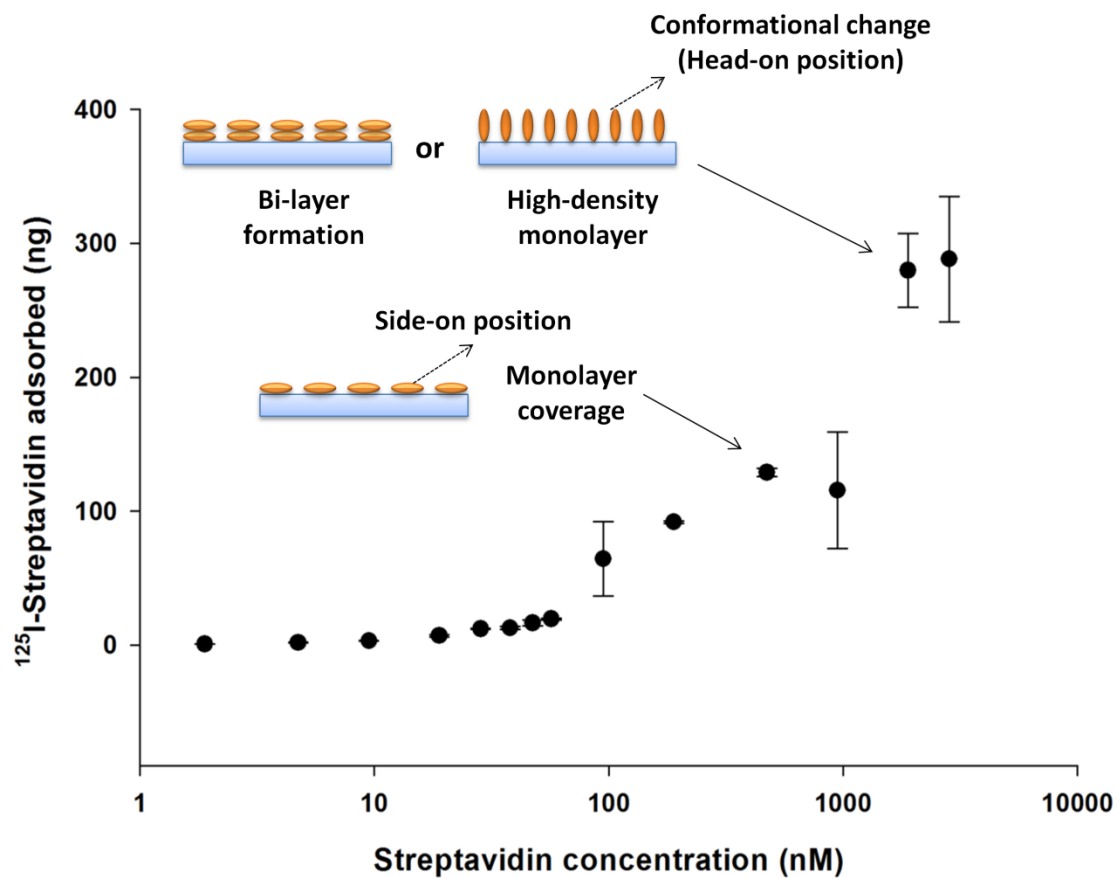


Figure 5-1: Extent of base layer coverage - streptavidin adsorption on base layer. Plot shows protein adsorption for various incubated streptavidin concentration, characterizing extent of protein coverage on base layer (Layer 1) available for LBL assembly growth.

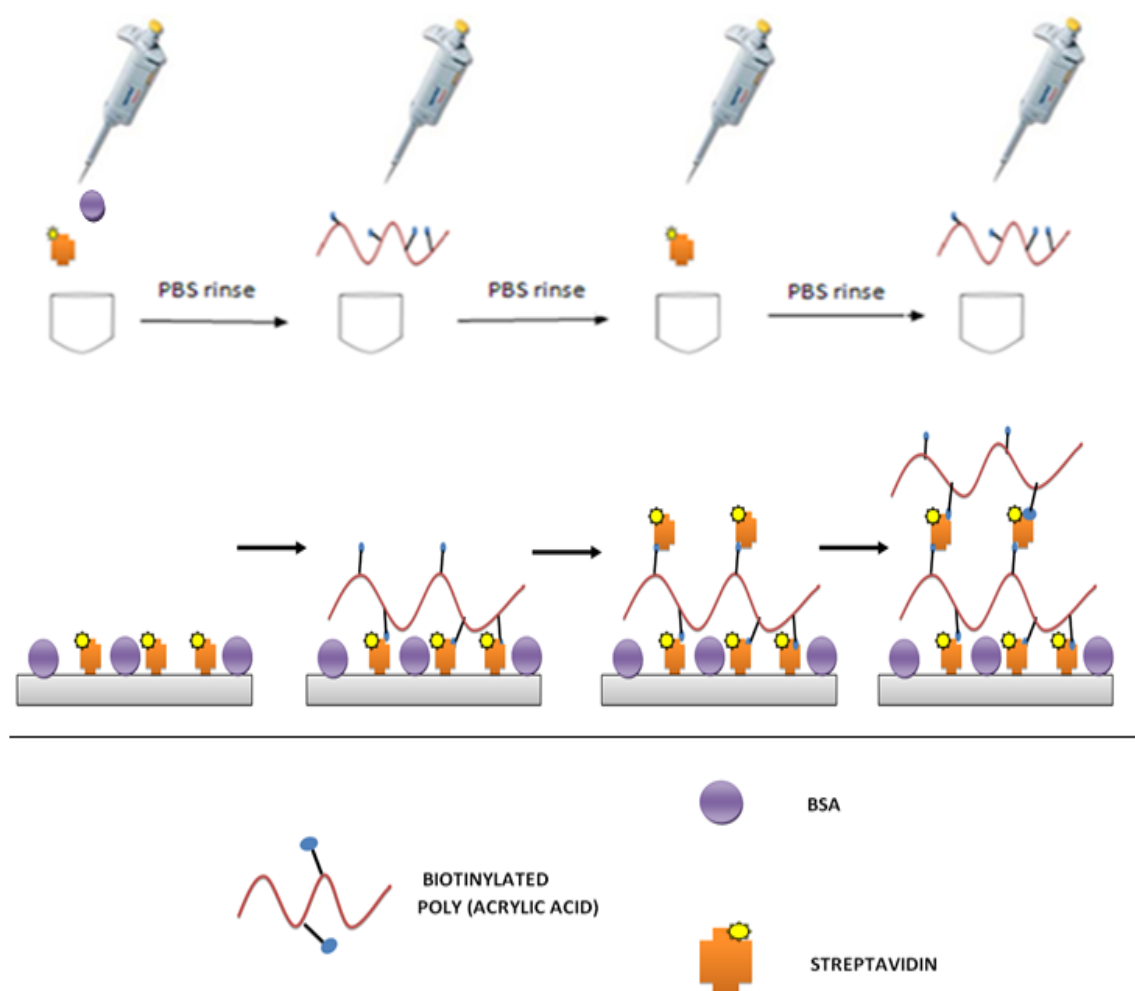


Figure 5-2: Scheme showing LBL assembly development using biotin-streptavidin affinity linkages by alternate additions of protein streptavidin and synthesized biotinylated PAA.

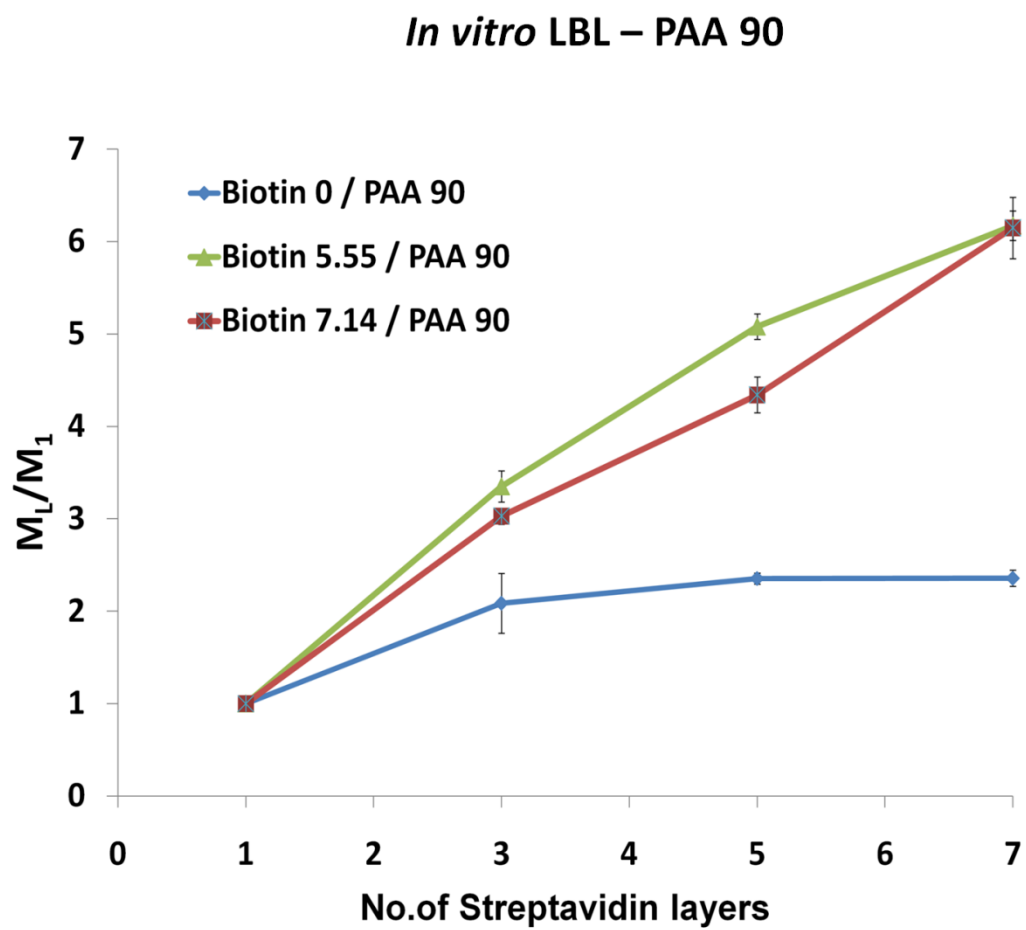


Figure 5-3: *In vitro* LBL assembly formation using high MW (90,000 Da) PAA of various extent of biotin conjugation.

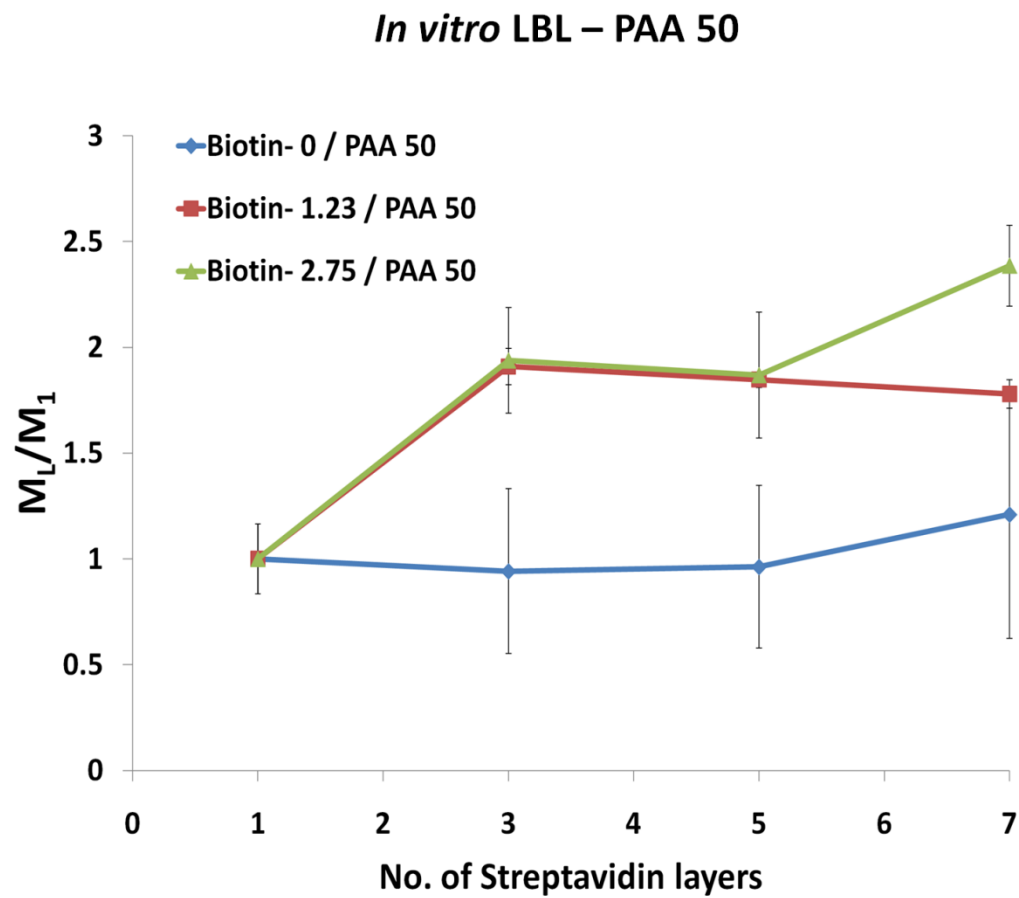


Figure 5-4: *In vitro* LBL assembly formation using mid-range MW (50,000 Da) PAA of various extent of biotin conjugation.

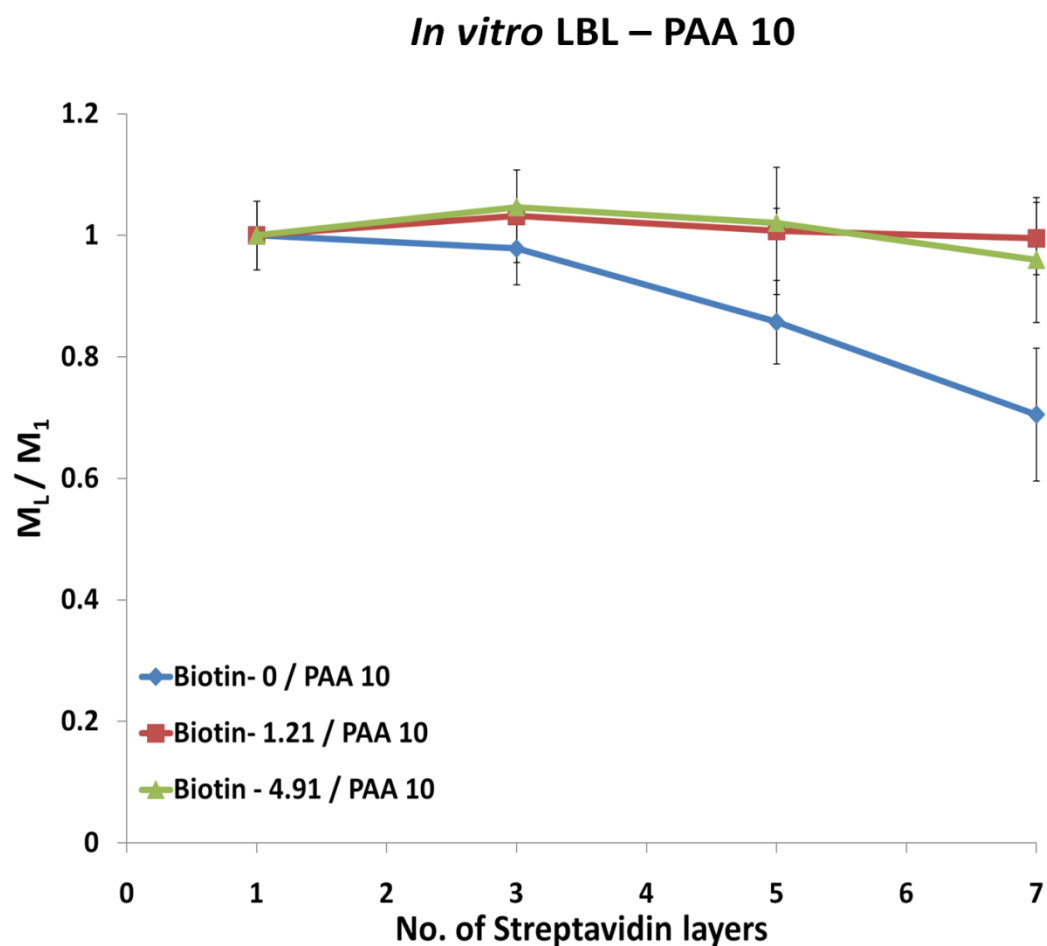


Figure 5-5: *In vitro* LBL assembly formation using low MW (10,000 Da) PAA of various extent of biotin conjugation.

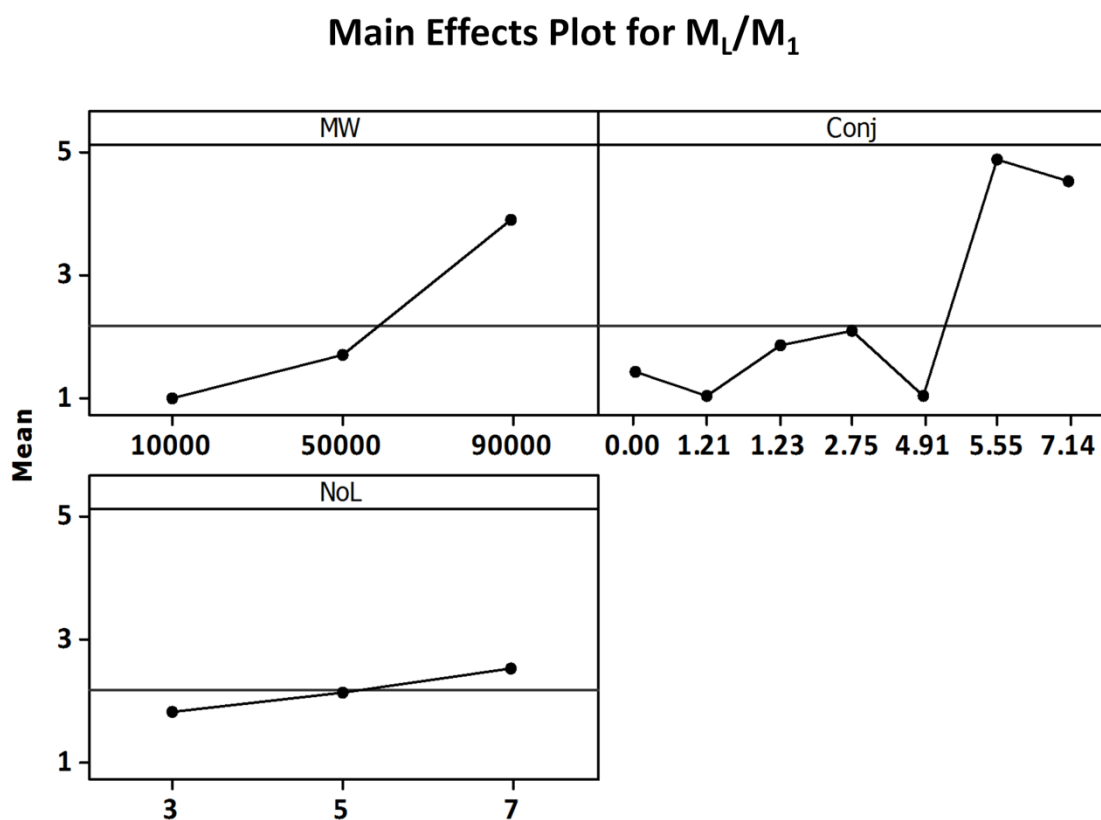


Figure 5-6: Main effects plot from ANOVA studying LBL factor effects on development of *in vitro* LBL assemblies.

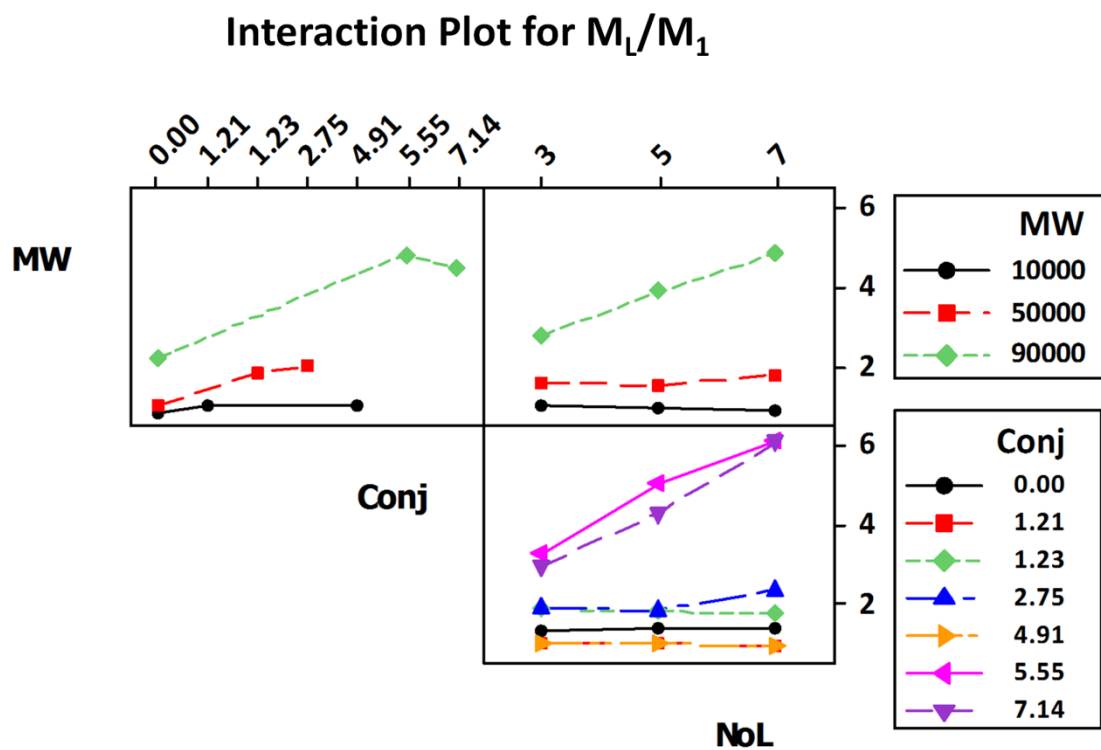


Figure 5-7: Interactions plot from ANOVA studying LBL factor effects on development of *in vitro* LBL assemblies.

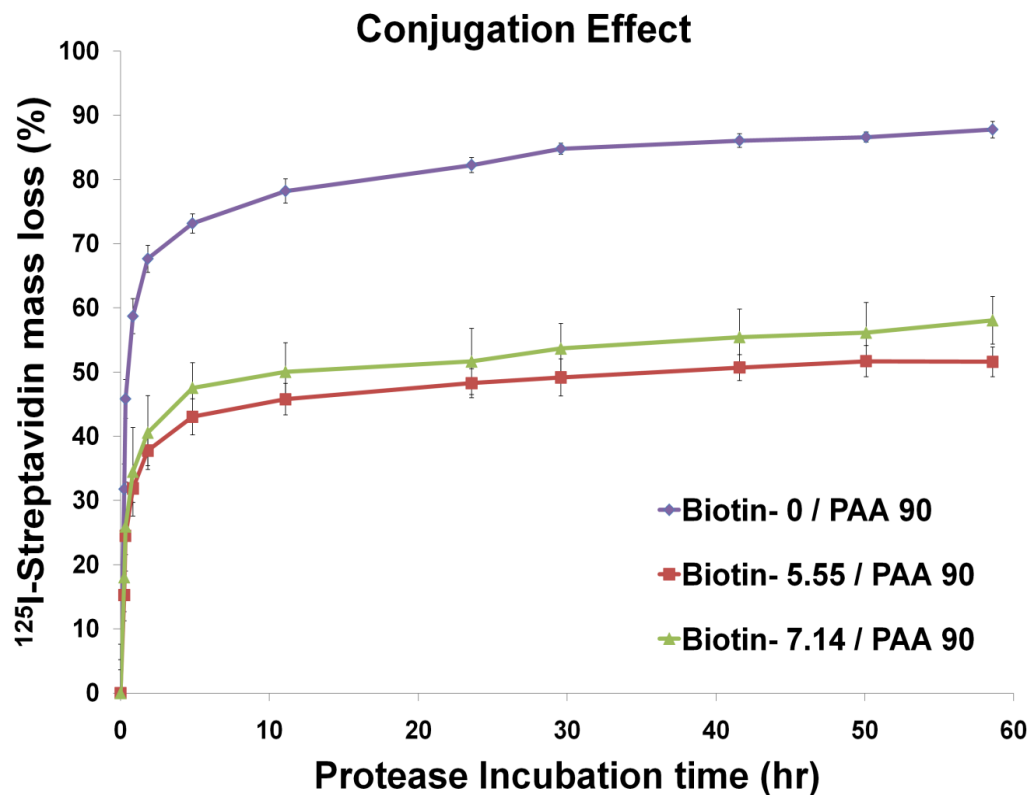


Figure 5-8: Effect of factor “extent of biotin conjugation” in LBL protease stability. Plot shows biotin conjugation effect by analyzing self-assemblies developed from polymer PAA of same MW (90,000 Da) with equal number of assembly layers (NoL=7) and through comparison with different extent of biotin conjugation. Similar analysis can be performed for same PAA 90 materials with different NoL in assemblies such as NoL=3 (see **Appendix figure A-3.1**), NoL=5 (see **Appendix figure A-3.2**). Also conjugation effects on mid-range MW (50,000 Da) polymeric materials (see **Appendix figure A-5.1, A-5.2, and A-5.3**) and in low MW (10,000 Da) polymeric materials (results not shown) was analyzed.

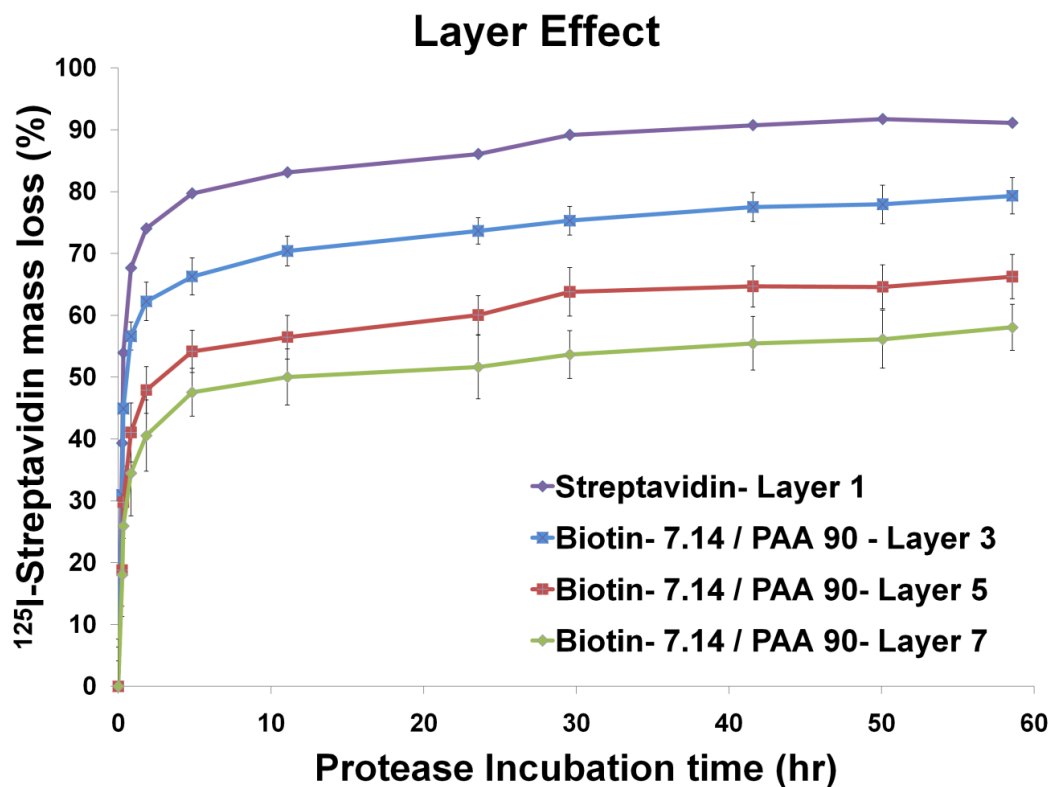


Figure 5-9: Effect of factor “number of assembly layers (NoL)” in LBL protease stability.

Plot shows layer effect in self-assemblies developed from high MW (90,000 Da) biotinylated PAA (Biotin-7.1 / PAA 90). Similar analysis was performed for the same PAA 90 materials with different conjugations such as Biotin=0 (see **Appendix figure A-4.1**), Biotin=5.55 (see **Appendix figure A-4.2**). Also layer effects on midrange MW (50,000 Da) polymeric materials (see **Appendix figure A-6.1, A-6.2 and A-6.3**) and in low MW (10,000 Da) polymeric materials ((results not shown) was analyzed.

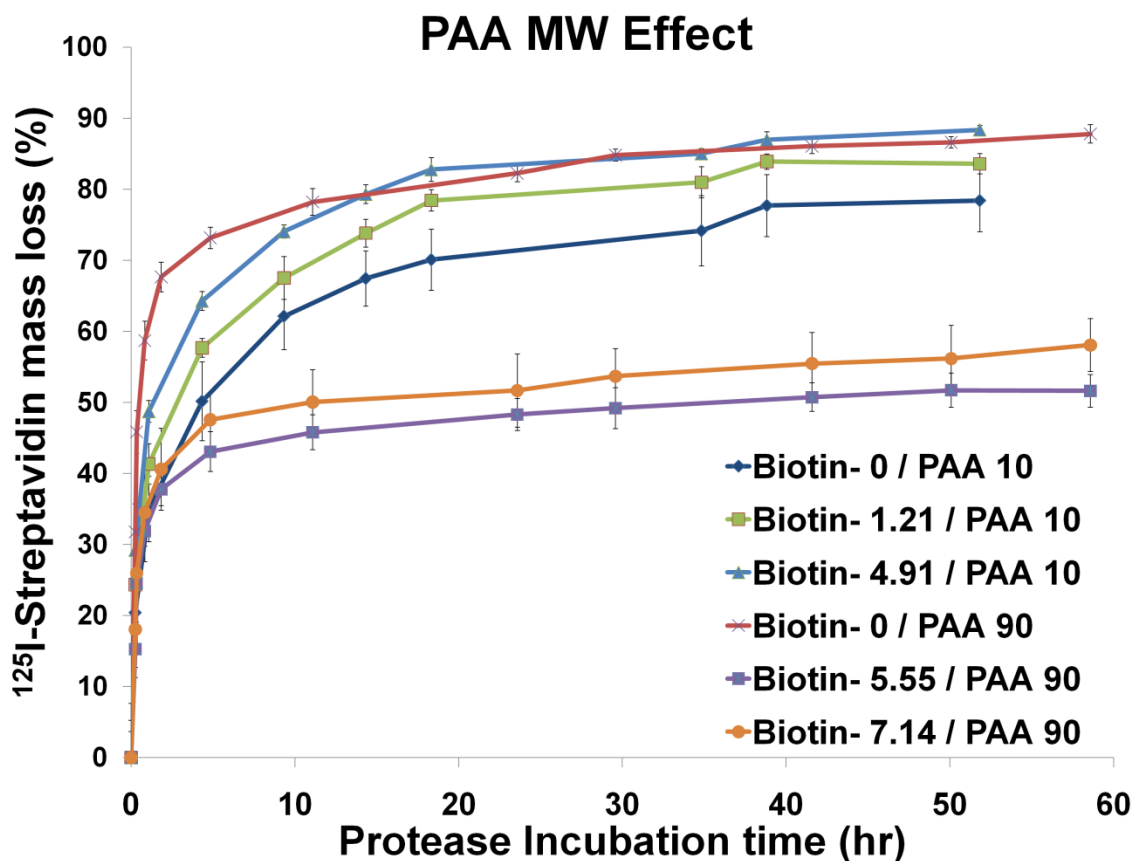


Figure 5-10: Effect of factor “polymer MW” in LBL protease stability. Plot shows MW effect by analyzing self-assemblies developed of equal number of assembly layers (NoL=7) and providing comparison with different MW (90,000/10,000 Da) PAA materials. Similar analysis can be performed with different number of LBL layers such as NoL=3 and NoL=5 (results not shown).

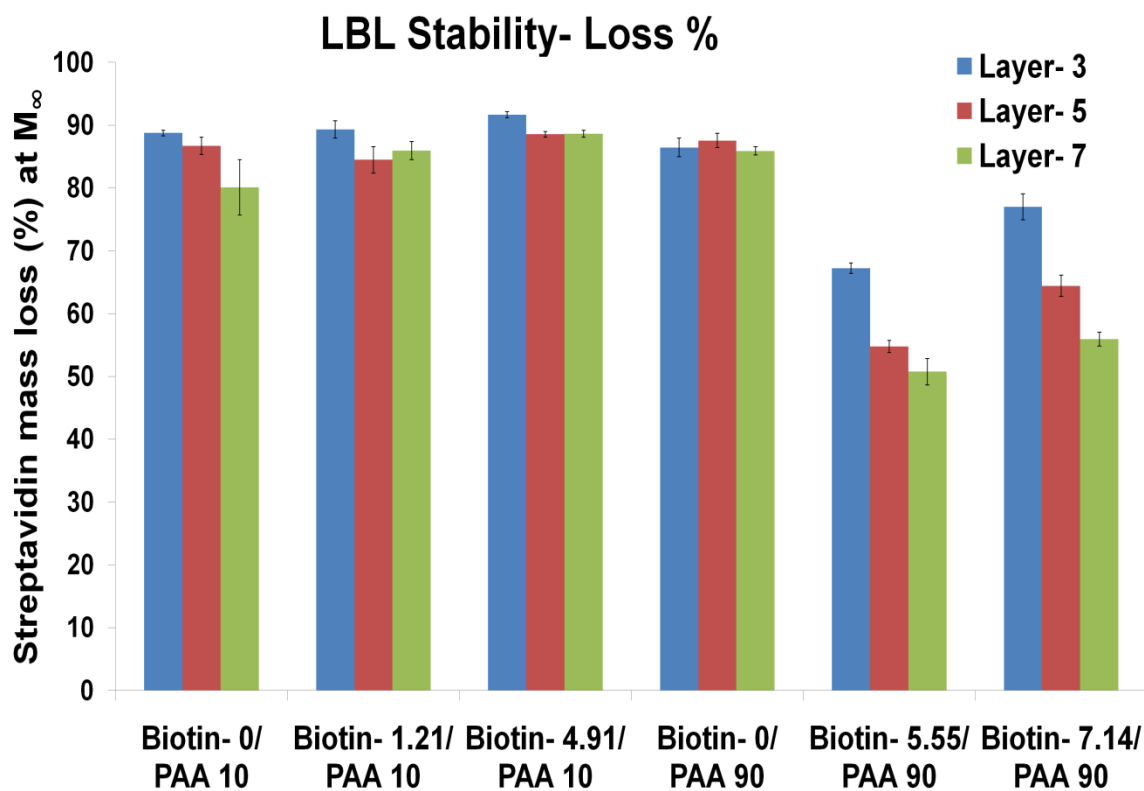


Figure 5-11: Streptavidin mass loss (%) at M_{∞} in LBL protease stability. Plot shows streptavidin mass loss (%) at M_{∞} obtained from protease stability plots of different LBL systems (MW / biotin conjugation / NoL). For similar analysis on midrange MW (50,000 Da) polymeric materials see **Appendix figure A-6.4**.

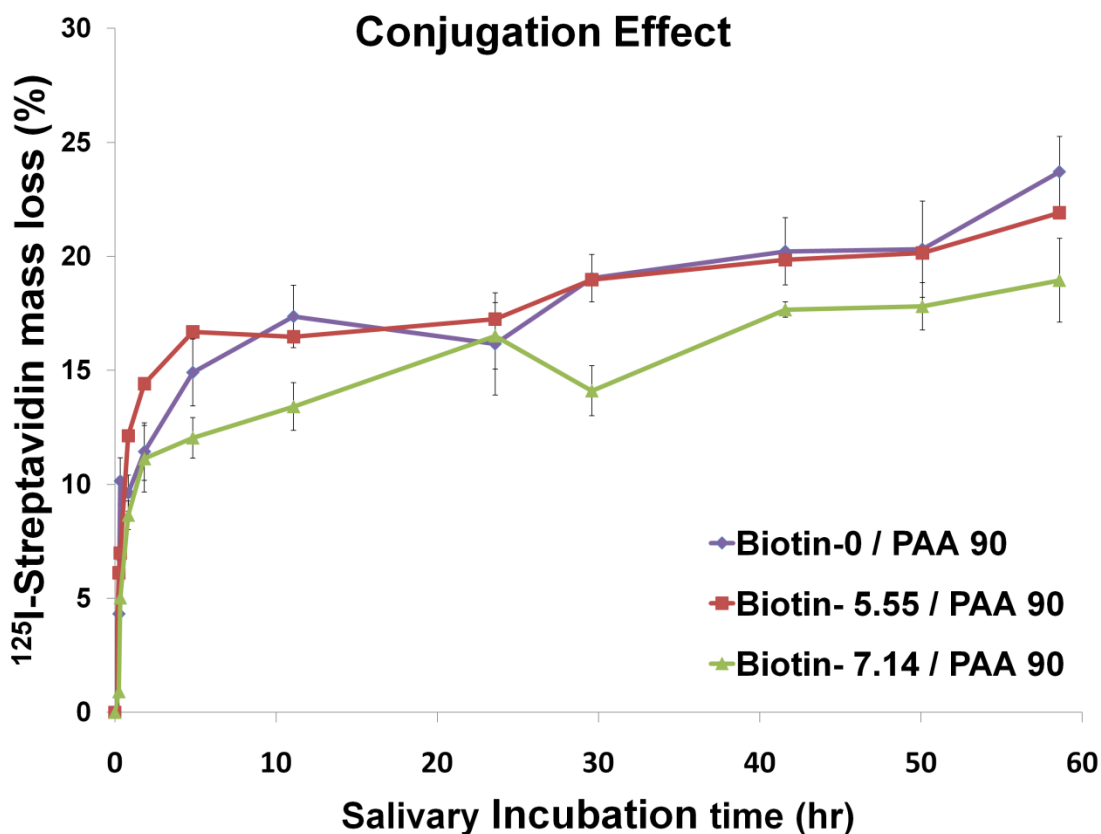


Figure 5-12: Effect on factor “extent of biotin conjugation” in LBL salivary stability. Plot shows biotin conjugation effect by analyzing self-assemblies developed from polymer PAA of same MW (90,000 Da) with equal number of assembly layers (NoL=7) and providing comparison with different extent of biotin conjugation. Similar analysis can be performed for same PAA 90 materials with NoL=3 (see **Appendix figure A-8.1**), NoL=5 (see **Appendix figure A-8.2**). Also conjugation effects on low MW (10,000 Da) polymeric materials was analyzed (results not shown).

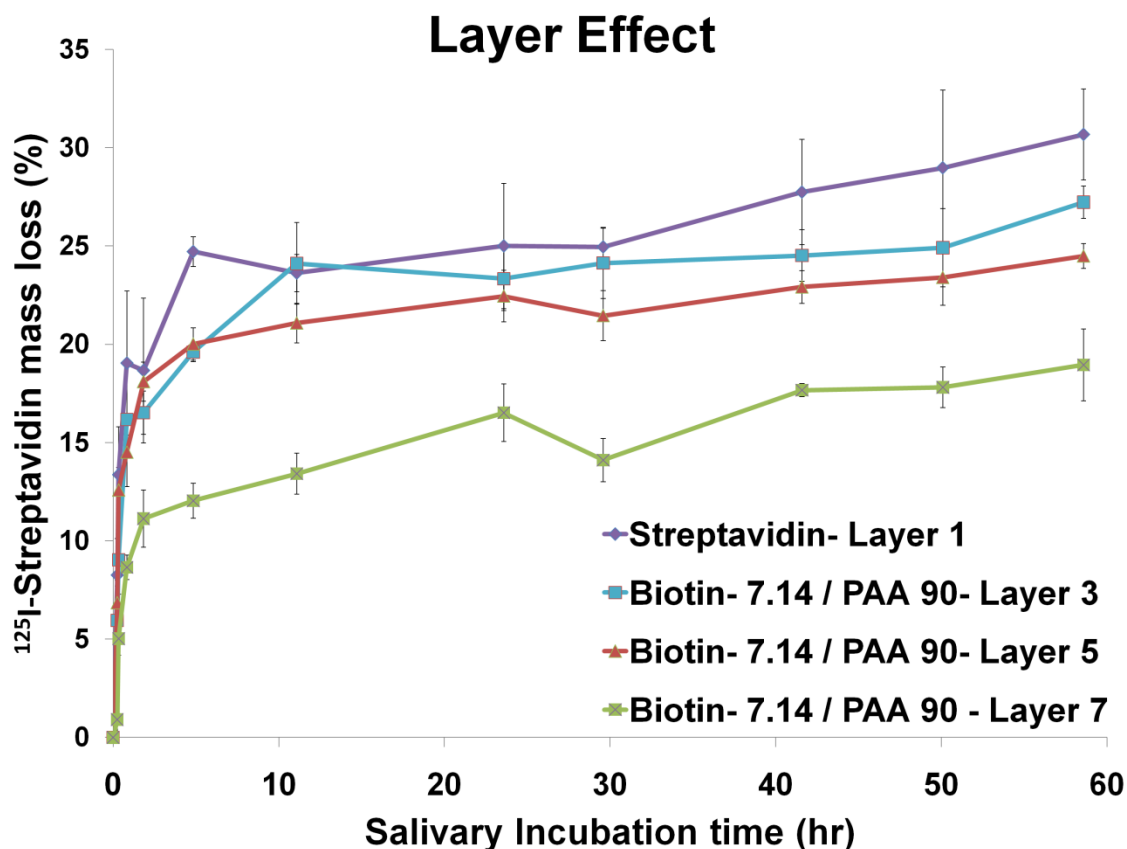


Figure 5-13: Effect on factor “number of assembly layers (NoL)” in LBL salivary stability.

Plot shows layer effect from LBL stability tests in self-assemblies developed from high MW (90,000 Da) biotinylated PAA (Biotin-7.1 / PAA 90). Similar analysis was performed for the same PAA 90 materials with different conjugations such as Biotin=0 (see **Appendix figure A-9.1**), Biotin=5.55 (see **Appendix figure A-9.2**). Also layer effects on low MW (10,000 Da) polymeric materials was analyzed (results not shown).

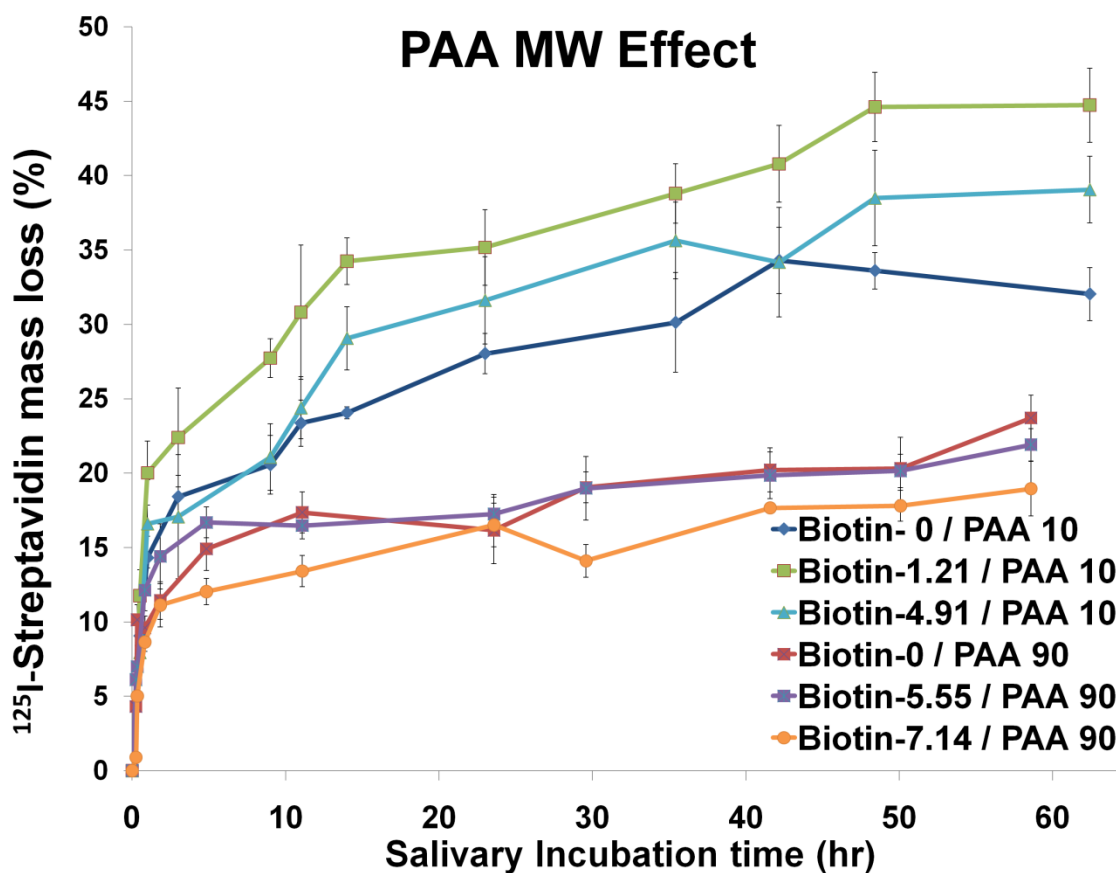


Figure 5-14: Effect on factor “polymer MW” in LBL salivary stability. Plot shows MW effect by analyzing self-assemblies developed of equal number of assembly layers (NoL=7) and providing comparison with different MW (90,000/10,000 Da) PAA materials. Similar analysis can be performed with different number of LBL layers.

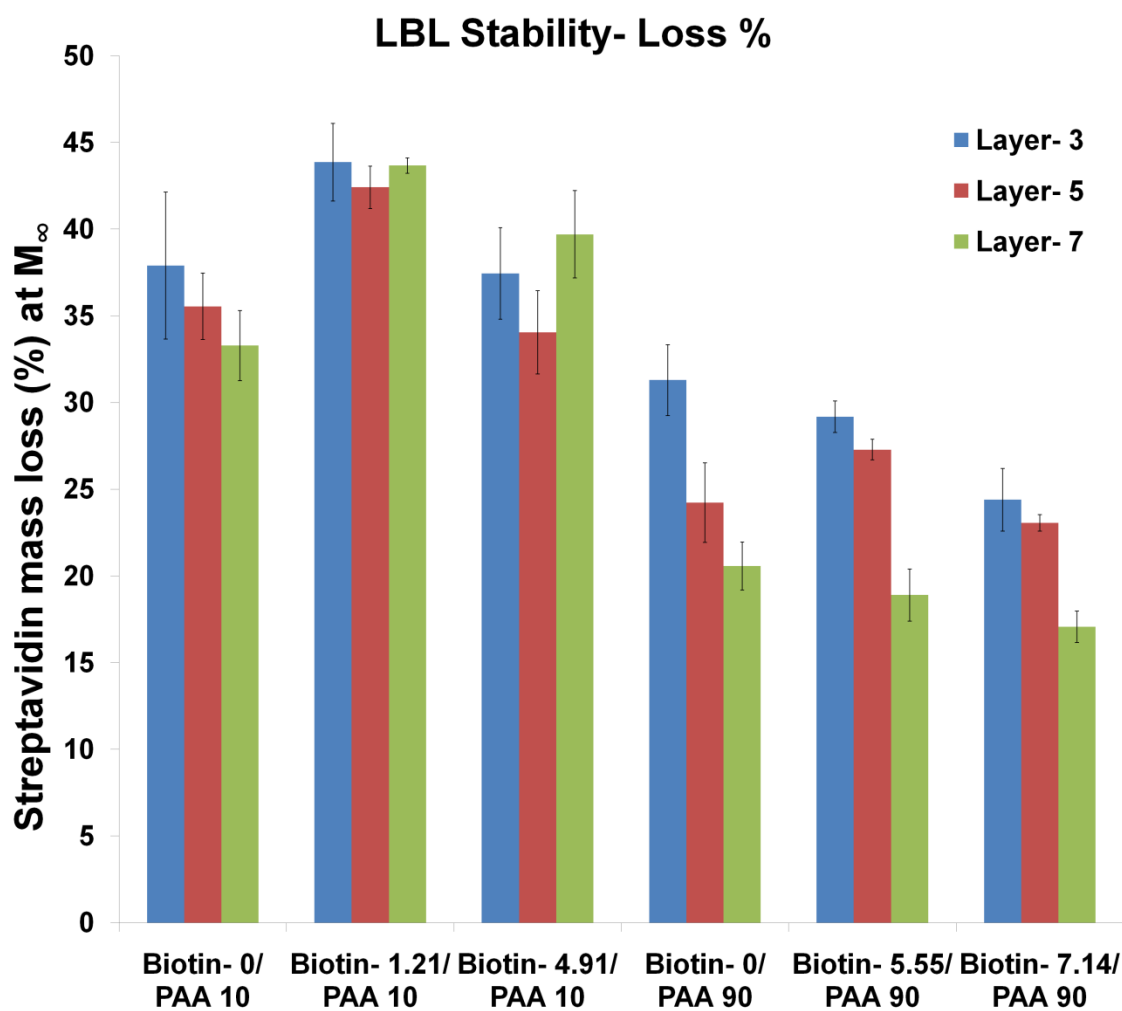


Figure 5-15: Streptavidin mass loss (%) at M_{∞} in LBL salivary stability. Plot shows streptavidin mass loss (%) at M_{∞} obtained from salivary stability plots of different LBL systems (MW / biotin conjugation / NoL).

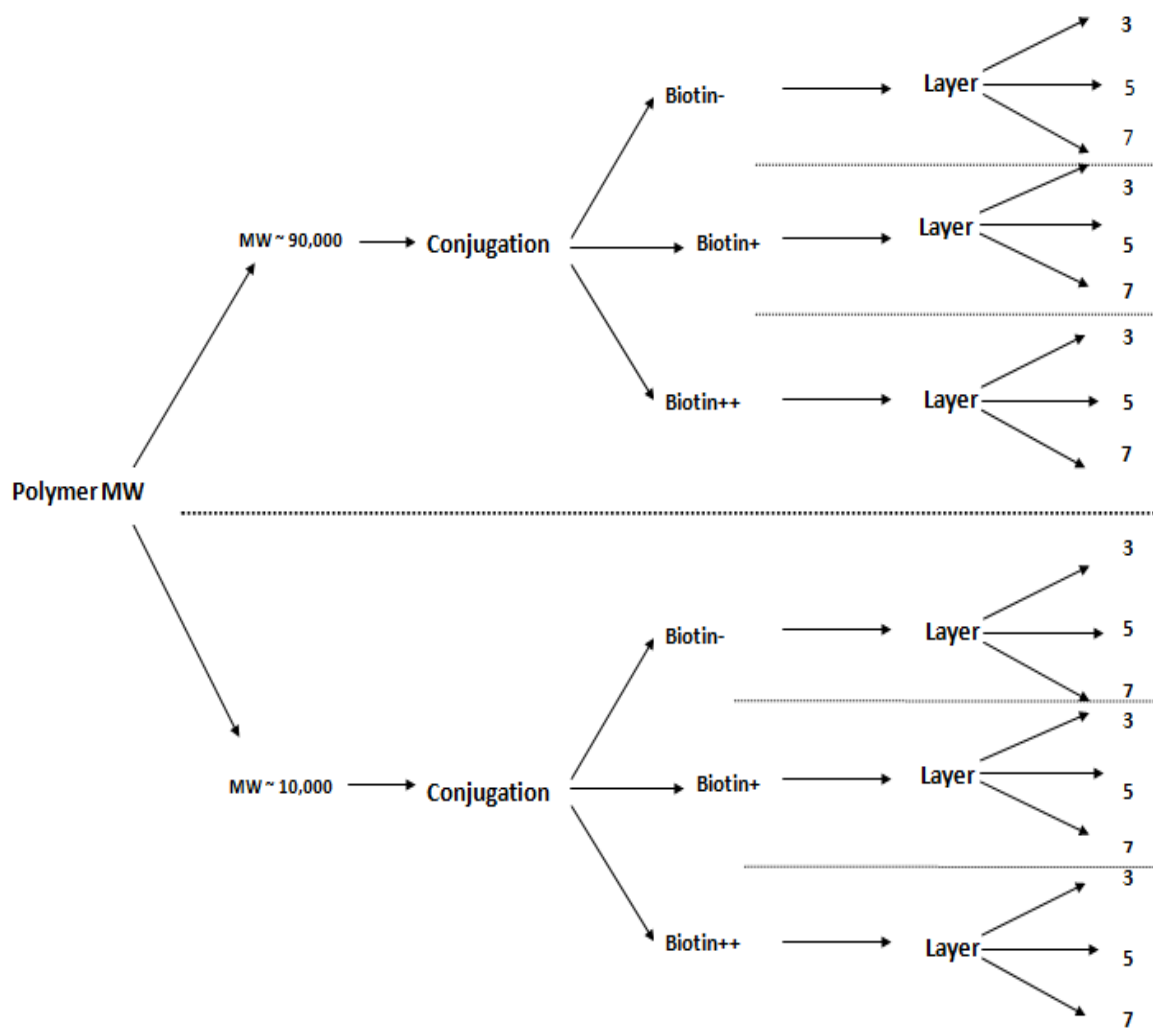


Figure 5-16: Scheme showing design used in 3-way nested ANOVA analysis which contains group (MW= 90,000 and 10,000), with subgroups (Biotin conjugation) and (NoL).

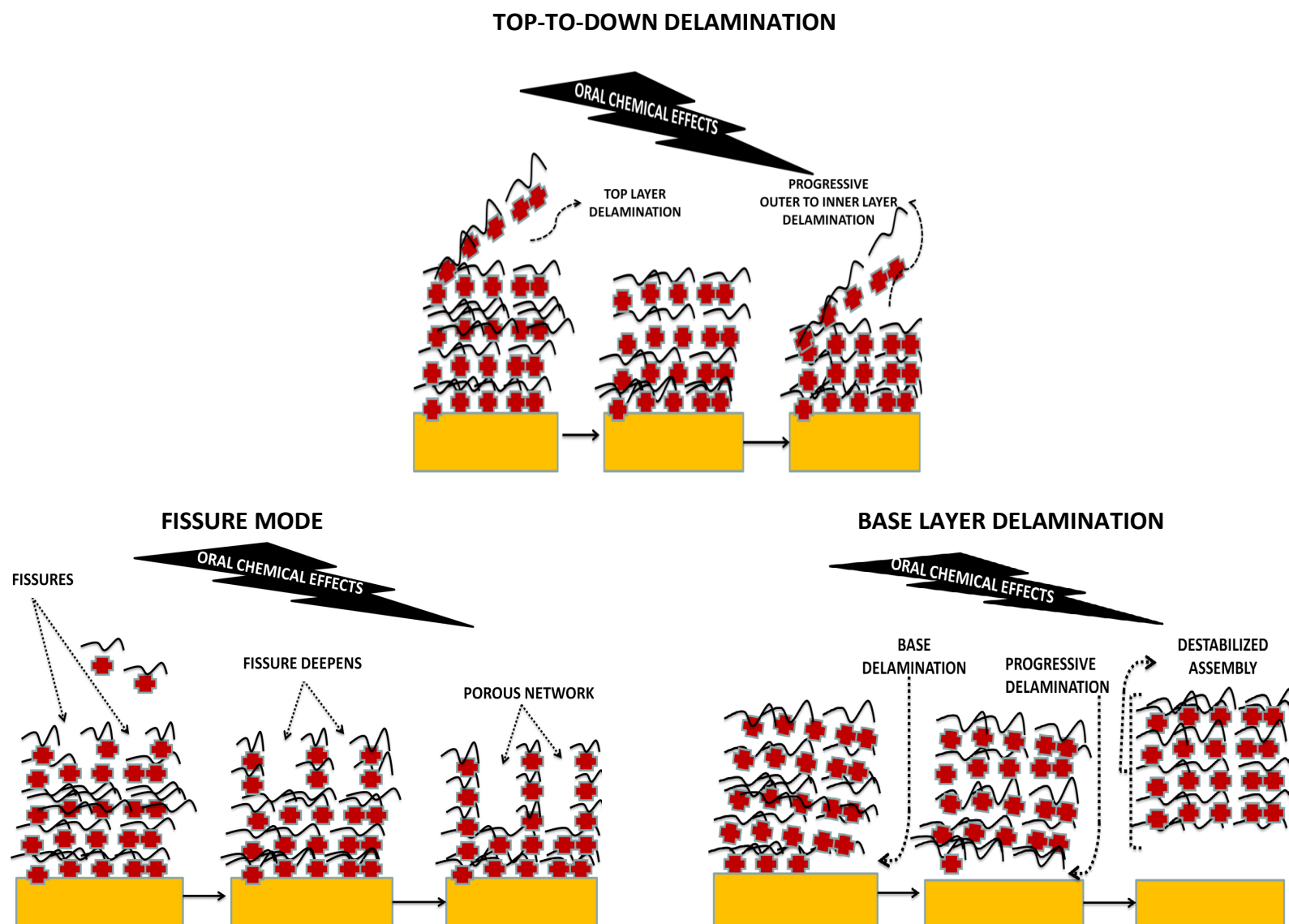


Figure 5-17: Possible LBL degradation mechanism under oral chemical effects.

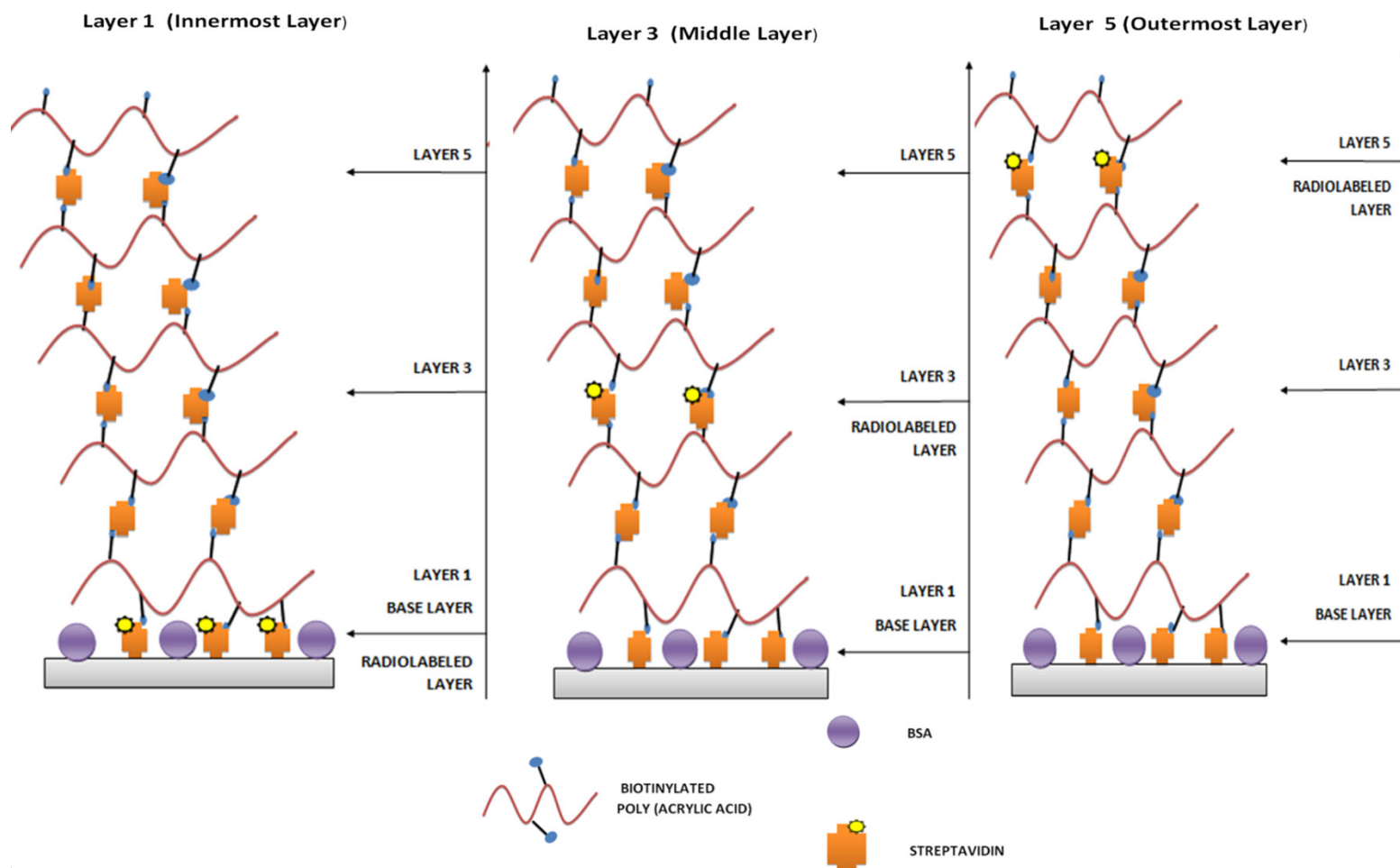


Figure 5-18: Scheme shows developed 5-layered LBL systems, to study degradation mechanism from innermost, middle and outermost layers by radiolabeling respective layers in self-assemblies 1, 3 and 5.

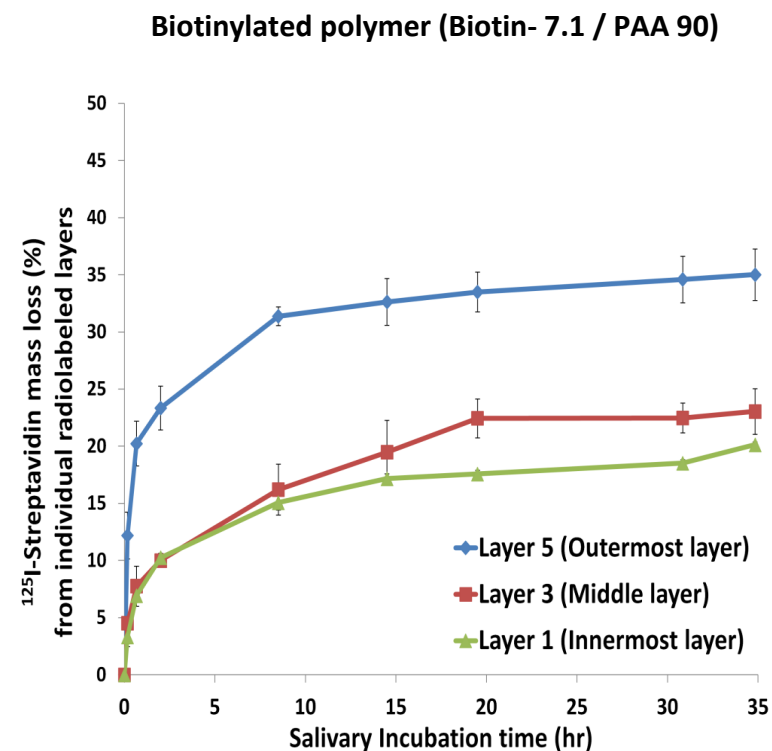
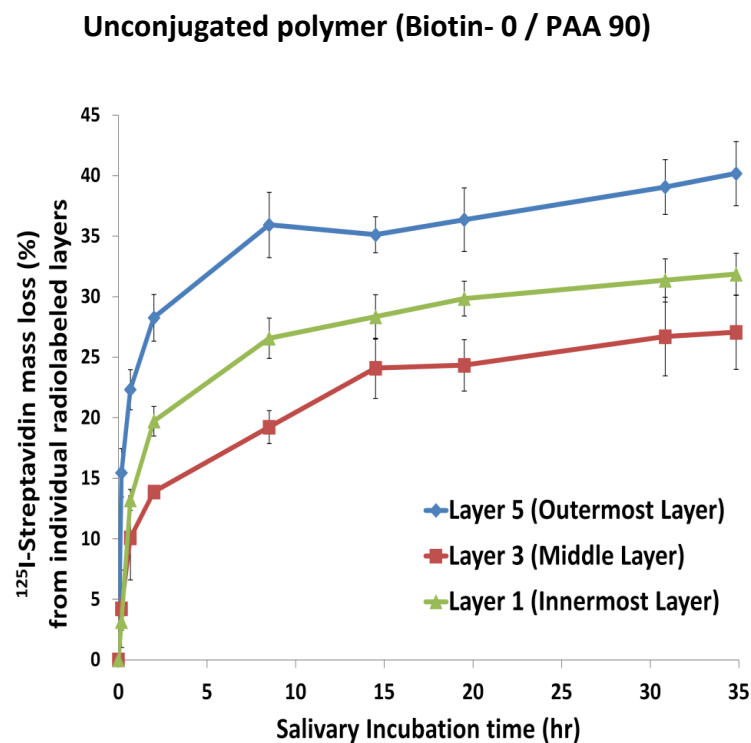


Figure 5-19: Results studying mechanism of *in vitro* LBL degradation. 5-layered assemblies were developed from high MW (90,000 Da) biotinylated PAA (Biotin-7.1 / PAA 90) and compared with unconjugated PAA (Biotin-0 / PAA 90) using mass loss (%) from individual radiolabeled layers.

CHAPTER 6. MECHANICAL STABILITY TESTS ON LAYER-BY-LAYER SELF-ASSEMBLIES

As a result of mandibular motions and abrasion from food, the oral mucosa is continually exposed to wear conditions. In an oral environment, it is expected that shear and abrasion forces are the main factors that influence LBL barrier destabilization. Hence, in testing LBLs barrier durability and wear resistance against intraoral abrasions, repeat contact barrier fatigue test were performed on *ex vivo* LBLs. Such adhesion testing is expected to be representative of *in vivo* conditions and provide a measure of LBLs performance in an intraoral mechanical environment. In evaluating LBLs mechanical stability, porcine skin patches were chosen as a model tissue substrate due to their structurally similar collagen arrangement to a human dermis [149, 150].

6.1. Materials and methods

6.1.1. *Ex vivo* LBL

Ex vivo LBL studies were developed on pig skin tissue. Porcine skin patches of dimension (5 mm x 5 mm) were used. Assembly development over tissue-protein base layer was carried out by alternating polymer/protein rinses, adopting the same procedure as described earlier for *in vitro* LBL studies. For *ex vivo* LBL studies, biotinylated PAA of MW 50,000 Da was used.

6.1.2. Mechanical stability tests on *ex vivo* LBL assemblies

Mechanical stability was studied on *ex vivo* LBL assemblies using a mechanical fatigue tester, Bose Electroforce (ELF 3300 test system) equipped with a 1 kg load cell. Porcine skin patches (16 mm diameter) were thawed to room temperature and rinsed

with PBS to avoid tissue dryness, emulating oral wounds. Tissues with dermal surfaces facing each other were mounted to loading platens using cyanoacrylate and fixed in place by applying a constant load.

In the adhesion tests, a compressive load of 3 N (305.8 g) was applied with a ramp rate of 10 g/sec under load control and was stabilized at the load for 1 minute. Under displacement control, pull off was conducted at a rate of 0.1 mm/sec until full tissue separation occurs. Adhesion tests were performed on tissues before (tissue trend) and after LBL development (LBL trend). LBL assembly was developed on tissue fixed to the lower platen using the same procedure as described earlier in *ex vivo* LBL studies, using cold streptavidin (non-radioactive) for the studies. In LBL fatigue resistance tests, contact loadings of 20-25 cycles (load–pull offs) were provided after LBL growth. During wear tests, tissue dryness was avoided by constant wetting with PBS between the cycles thereby maintain similar tissue working conditions. By maintaining constant tissue wetting conditions, the variation caused by tissue dryness between loading cycles were minimized. Tissue trend was obtained prior to assembly development to ensure that variations in tissues on loadings were not significant. Contact loadings of a minimum of 10 cycles were performed to determine this trend.

6.2. Results and Discussion

6.2.1. *Ex vivo* LBL

In order to determine the ability to develop LBLs on tissue and to validate future *ex vivo* LBL tests, tissue assembly development using PAAs of MW 50,000 Da (PAA50)

was studied. Porcine skin patches of equal dimensions were used during studies, thereby maintaining a consistent surface area. Here, LBL assemblies were developed over tissue-protein base layer; utilizing the non-specific protein interactions with tissue surface receptor sites to form tissue-protein complex in base layer [151, 152]. From *ex vivo* LBL studies, biotinylated polymers (Biotin- 2.75 / PAA 50 and Biotin- 1.23 / PAA 90) showed a significantly better self-assembly ability with higher assembly layers (NoL= 4 & 5) as when compared to unconjugated polymer (Biotin-0 / PAA 90) (**Figure 6-1**). In 5-layered LBL assemblies (NoL=5) for biotinylated PAA materials (Biotin-1.23/PAA 50, Biotin-2.75/PAA 50), the streptavidin mass per tissue surface area was 3.18 mg/m² and 3.62 mg/m², respectively, which was 31-49 % greater adsorbed protein mass compared to the unconjugated PAA material (Biotin-0/PAA 50). For unconjugated polymer (Biotin-0/PAA 50) there was a relatively weak streptavidin mass increase suggesting a non-specific binding during assembly growth verified through control comparison with extent of protein adsorption in tissue without any polymer addition during assembly growth. From *ex vivo* studies, the ability to form and thereby to distinguish tissue-LBL assemblies even with relatively low assembly layers (NoL=5) from conjugated polymers was deduced.

6.2.2. LBL mechanical stability

In studying LBL durability and its wear resistance, adhesion tests were performed on developed *ex vivo* LBL assemblies, scheme shown in **Figure 6-2**. For developing *ex vivo* assemblies of better stability during mechanical tests, the key parameters as determined from earlier chemical stability studies and multivariate

analyses were utilized. The earlier results deduced LBLs of conjugated materials with increased NoL and higher MW contributed to better LBL stability. Hence during adhesion tests, *ex vivo* LBLs of relatively high polymeric MW (50,000 / 90,000 Da) and increased assembly layers (NoL=14) were developed for different extent of biotin conjugation.

Adhesion tests were studied through rendered physical barrier effects of multilayered polymeric assemblies. This barrier effect from *ex vivo* LBL was measured through its extent of adhesive suppression in surrounding tissue (tissue mounted on upper platen). In analyzing LBL barrier durability, a repeat contact barrier fatigue model was used, where the developed *ex vivo* assemblies was subjected to repeated mechanical loading cycles (load-pull offs).

During adhesion tests, for each load-pull off cycle, a work of separation (WoS) was found through area under the curve in load vs. displacement plots, shown in inset **Figure 6-3**. On providing repeated loading cycles on tissues prior to assembly development, a “tissue trend” was obtained which accounts for possible tissue variations on repeated loadings. On developing *ex vivo* LBL over this studied tissue, repeated contact loading was provided to give a “tissue LBL trend” which evaluates LBLs wear resistance, shown in **Figure 6-3**.

From adhesion test results, biotinylated LBLs (Biotin- 7.1 / PAA 90 & Biotin- 2.75/ PAA 50) were able to significantly reduce tissue surface adhesion by its barrier property reflected from its decreased peak area, as shown in **Figure 6-4**. In analyses from wear

tests, these biotinylated polymers were able to maintain their physical barrier effect by remaining non-adhesive to surrounding tissues, even after numerous contact loadings. This physical barrier effect from biotinylated materials (Biotin- 7.1 / PAA 90 & Biotin- 2.75/ PAA 90) is shown in **Figure 6-4**, by its decreased work of separation (WoS) and with its minimal increase of WoS on providing repeated loading cycles (expressed in terms of tissue normalized WoS). A control from LBL of unconjugated high MW (90,000 Da) polymer (Biotin-0/PAA 90) resulted in poor barrier stability with an increased polymer adhesion to surrounding tissues. This enhanced adhesion in unconjugated polymers was likely a result of protein/polymer charge based interactions on surrounding tissue caused by its weakly formed layers. With LBLs of increased biotin conjugation better physical barrier was formed (Biotin-7.1 > Biotin-2.71 > Biotin-0) thereby demonstrates the effect of biotin conjugation in formulating durable barriers. Thus biotinylated polymers of higher MW and NoL yielded better barrier stability.

Ex vivo Tissue LBL – PAA 50

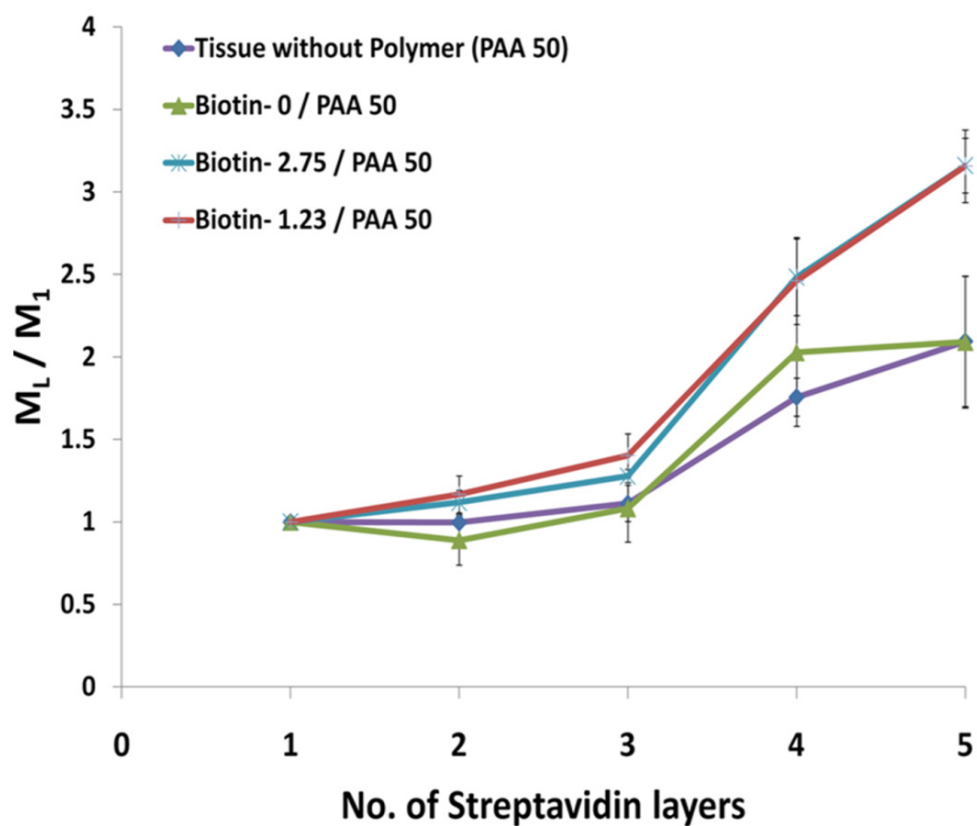


Figure 6-1: *Ex vivo* tissue LBL assembly formation. Plot shows LBL assembly formation from PAA of MW (50,000 Da) with biotin conjugation ([Biotin++] =2.75 and [Biotin+] =1.23).

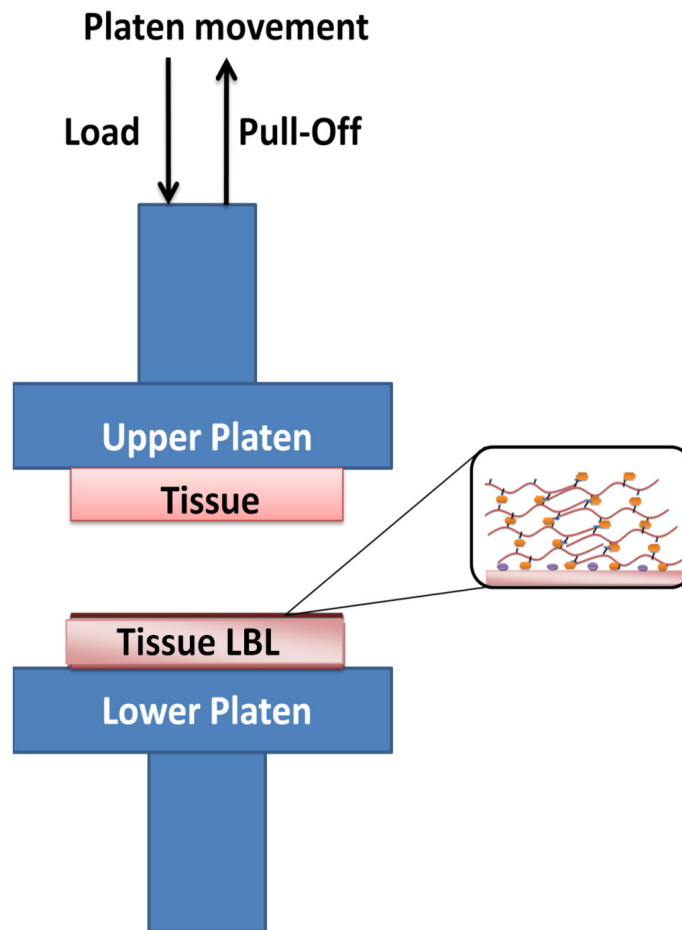


Figure 6-2: Scheme for *ex vivo* LBL adhesion testing. Developed tissue-LBL assemblies (lower platen) were subjected to adhesion loading through load-pull off cycles.

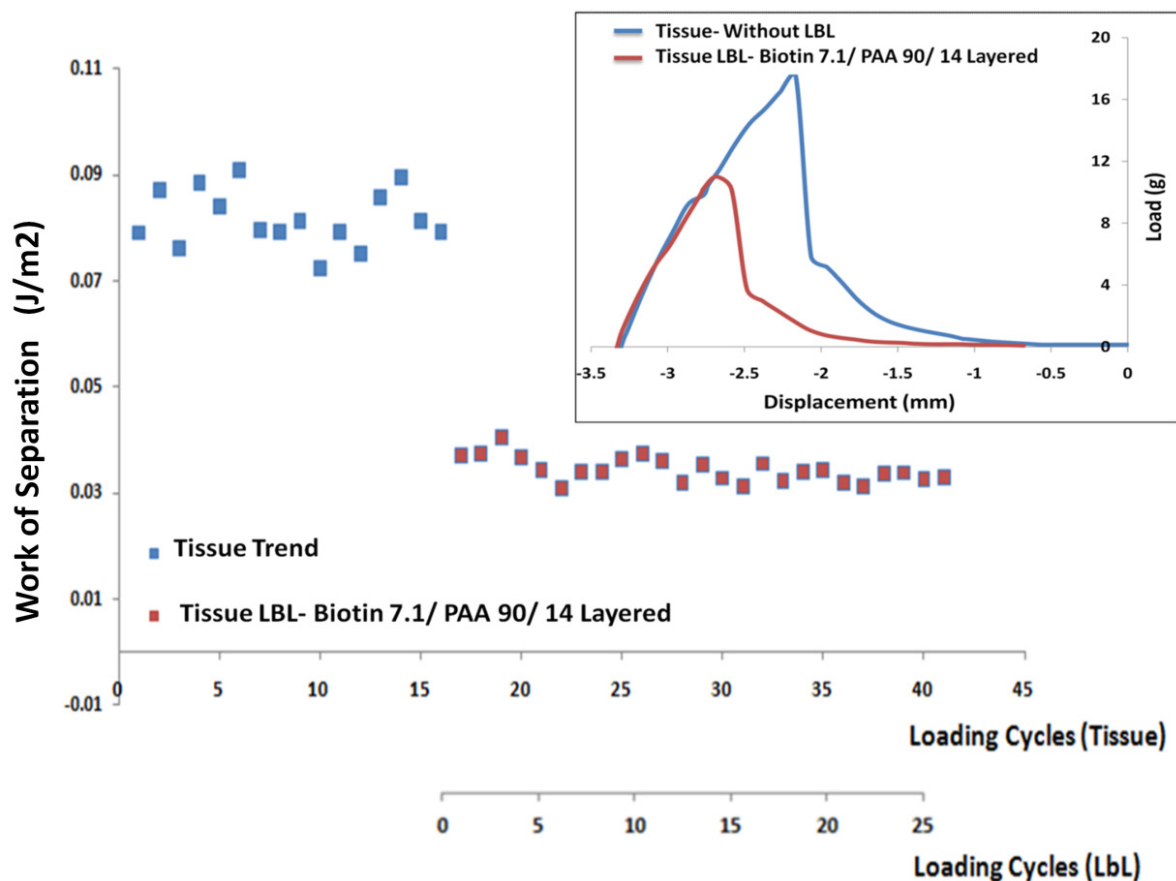


Figure 6-3: Repeated contact barrier fatigue tests. By providing repeated contacts of loading cycles (load-pull offs), adhesion trends were studied before and after LBL development, thereby studying LBL wear resistance. Plots show significant wear resistance from LBL barriers developed from biotinylated PAA (Biotin- 7.1/PAA 90/14 Layers) with reduced work of separation (WoS) on repeated LBL loading cycles. Inset plot shows load vs. displacement curves collected during adhesion tests before and after LBL development using biotinylated PAA (Biotin-7.1/PAA 90/14 Layers). Decrease in adhesion was shown by reduced WoS (area under the curve).

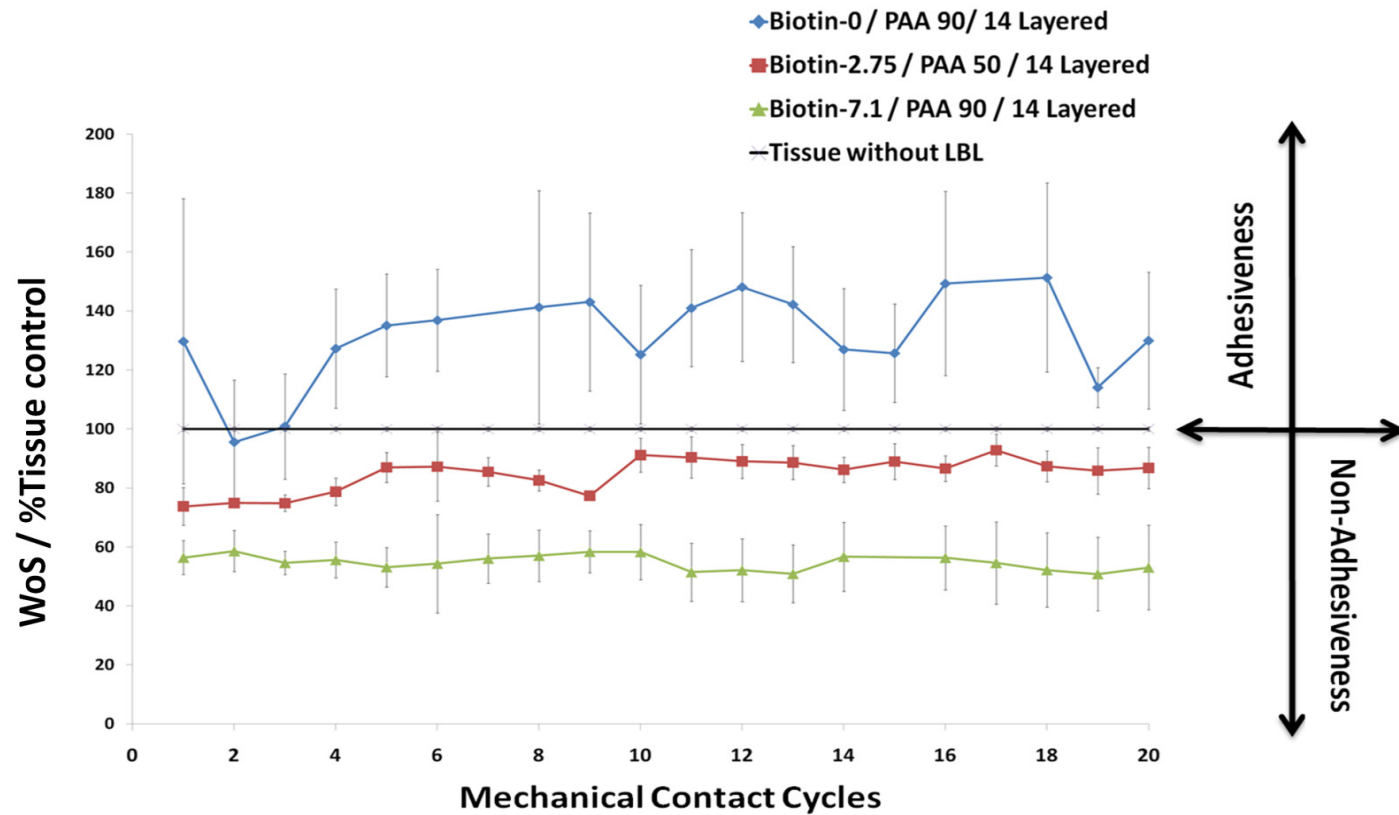


Figure 6-4: Summary of results from mechanical testing. 14 layered LBL assemblies developed from various PAA material (unconjugated (Biotin-0/PAA 90), (Biotin 2.75/PAA 50) and (Biotin 7.1/PAA 90)) were subjected to repeated loading cycles. The extent of adhesion on repeated contact was shown by tissue normalized work of separation (WoS / % tissue control). A hypothesis test using paired t-test was carried out on all LBL loadings trend and was found significant with $P < 0.01$.

CHAPTER 7. CONCLUSION AND FUTURE STUDIES

In this research, the ability to develop affinity based multilayered polymeric self-assembled multilayers was demonstrated. From chemical stability results and through explorative multivariate analysis, relationship between polymer properties and barrier function were obtained. LBLs of biotinylated polymers with higher MW and increased number of assembly layers demonstrated improved barrier properties. By evaluating LBL mechanical stability using a barrier fatigue model it was found LBLs of biotinylated polymers resulted in a durable barrier with excellent wear resistance. Thus, the affinity based multi-layered polymeric assemblies with their stable barrier property offer a potential regenerative treatment strategy for oral wounds.

With promising LBL stability results, the future study will be directed towards integrating therapeutic function to these stable multilayered assemblies. Potential future work involve drug loading studies analyzing the capping effects of overlaying polymer network and optimizing the zone of drug loading within assemblies studying their drug release kinetics. With current better understanding in pathogenesis of OM, it was well documented in the literature [39], the active role played by reactive oxygen species (ROS) in disease initiation. Hence, it was hypothesized LBL assemblies with their incorporated antioxidant nanoparticles (drug loadings) can formulate into an effective drug delivery system. Thus with this proposed treatment strategy a better tunable barriers which contribute to drug protection and thereby modulate drug release kinetics can be achieved.

APPENDIX

A.1. Base layer protein adsorption kinetics

Method: Radiolabeled streptavidin (541 nM) under un-agitated conditions was held for different incubation time in RIA plates and protein bound to the plates were measured after removing the unbound streptavidin using repeated PBS rinses (atleast 3).

Discussion: Under static conditions, monolayer coverage was obtained by providing an incubation time of 1 hour. Protein mass that resulted in monolayer coverage and its kinetics was obtained through this study. As expected, the mass of streptavidin required to form monolayer was consistent with the earlier conducted base layer protein adsorption studies, a result due to same surface area availability for protein adsorption.

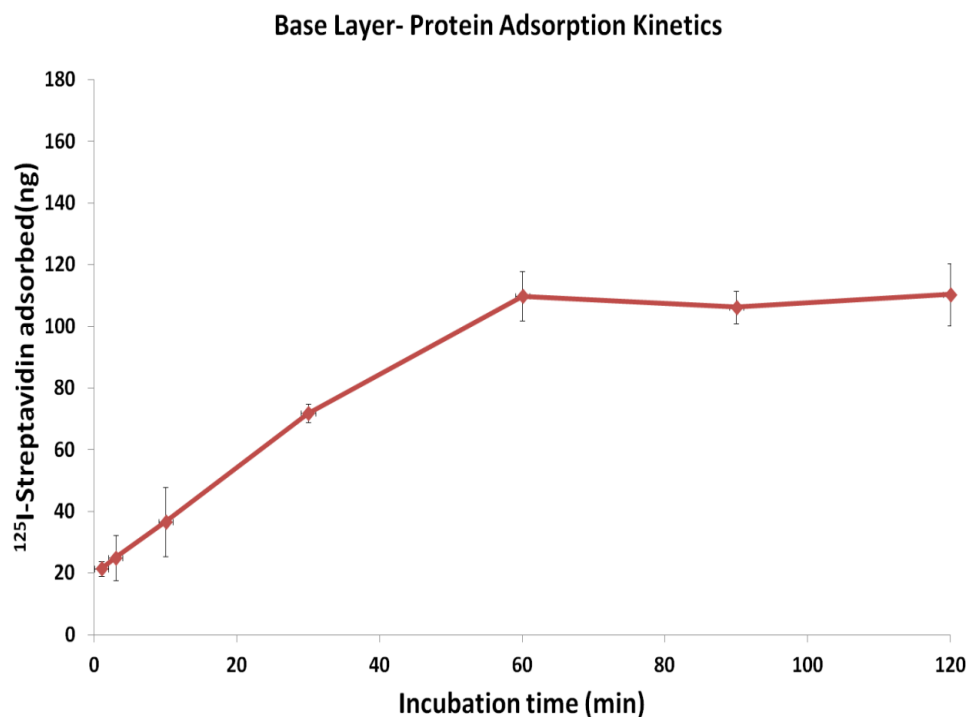


Figure A-1: Base layer adsorption kinetics - Plot shows kinetics of base layer streptavidin adsorption for various incubation times in RIA plates

A.2. Multivariate analysis on *in vitro* LBL assembly development (uncertainty plots)

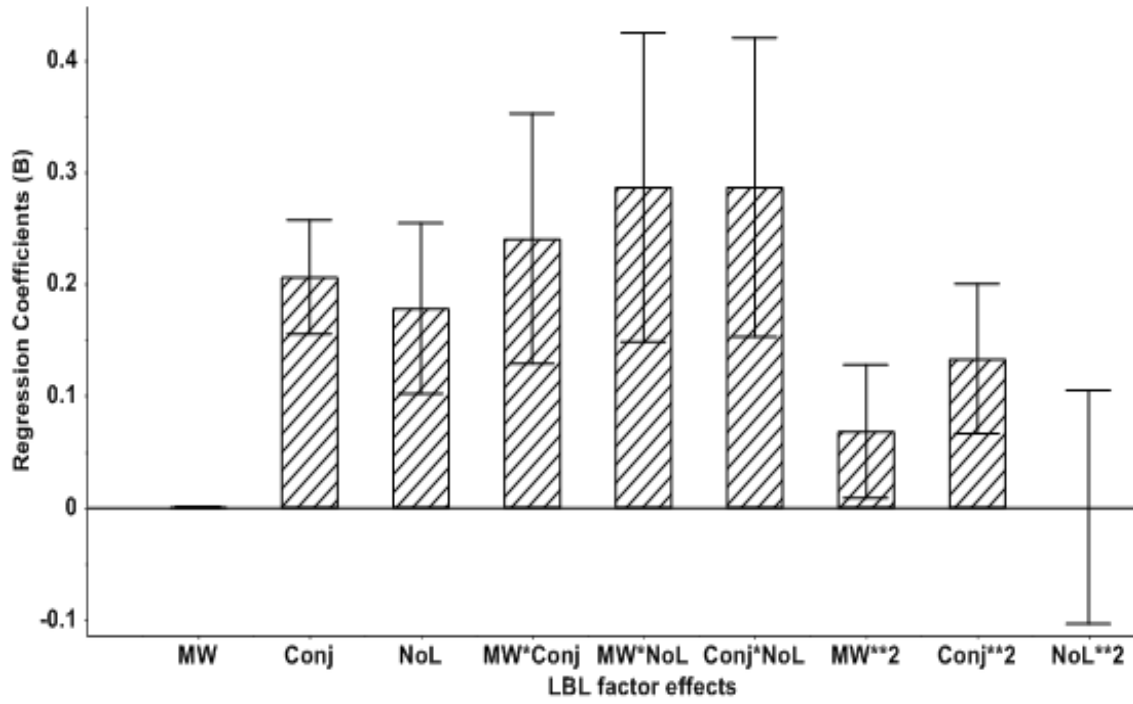


Figure A-2: Principal component regression analysis on *in vitro* LBL response variable (M_L/M_1) to study effects of various LBL factors (MW, conjugation and NoL). Bar plot shows principal component regression (PCR) analysis on regression coefficient (B) for various LBL factor effects, while considering response variable (M_L/M_1) during analysis. Results of test of significance on regression coefficient (B) (obtained from PCR analysis) was performed by uncertainty tests and factor effects were considered insignificant if uncertainty limits crosses the zero axes.

A.3. Protease stability on LBLs of high MW polymers studying conjugation effect

A.3.1. PAA (MW 90,000 Da) materials - conjugation effect in 3-layered assemblies

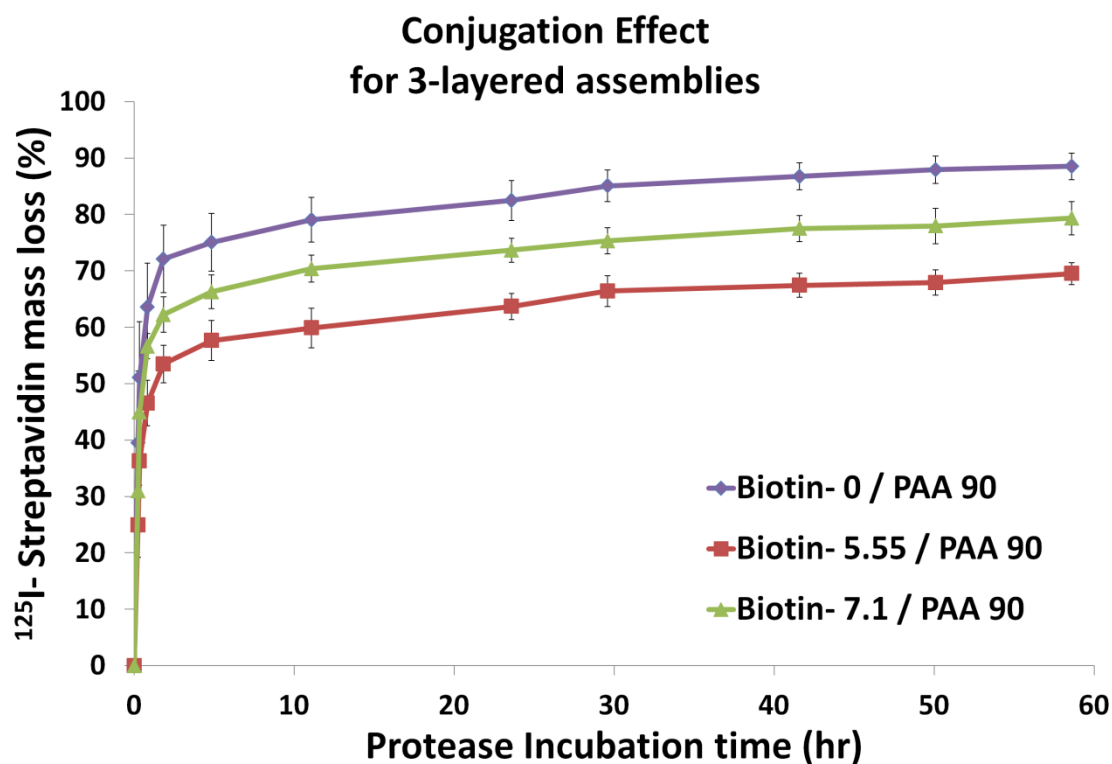


Figure A-3.1: LBL protease stability. Effect of factor “extent of biotin conjugation” from 3-layered assemblies (NoL=3) developed of high MW (90,000 Da) polymer with varying extent of biotinylation (Biotin- 0/5.55/7.1).

A.3.2. PAA (MW 90,000 Da) materials - conjugation effect in 5-layered assemblies

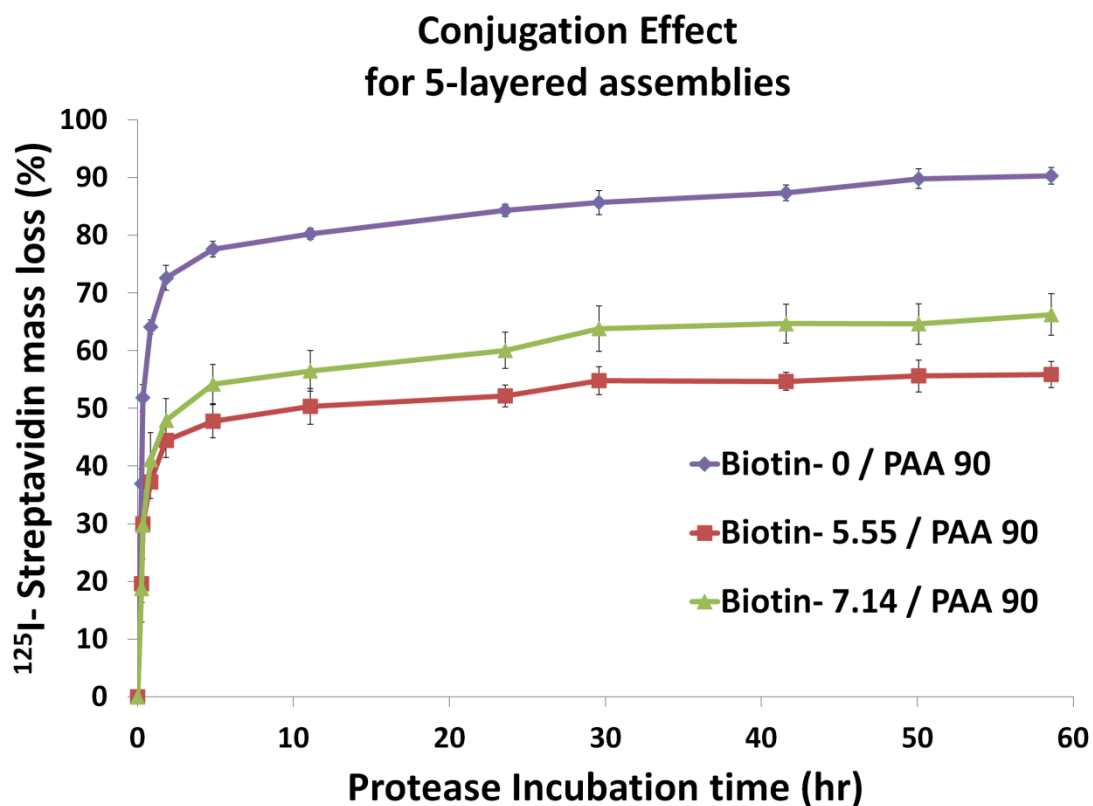


Figure A-3.2: LBL protease stability. Effect of factor “extent of biotin conjugation” from 5-layered assemblies (NoL=5) developed of high MW (90,000 Da) polymer with varying extent of biotinylation (Biotin-0/5.55/7.1).

A.4. Protease stability on LBLs of high MW polymers studying layer effect

A.4.1. PAA (MW 90,000 Da) materials - layer effects in LBLs of unconjugated polymer

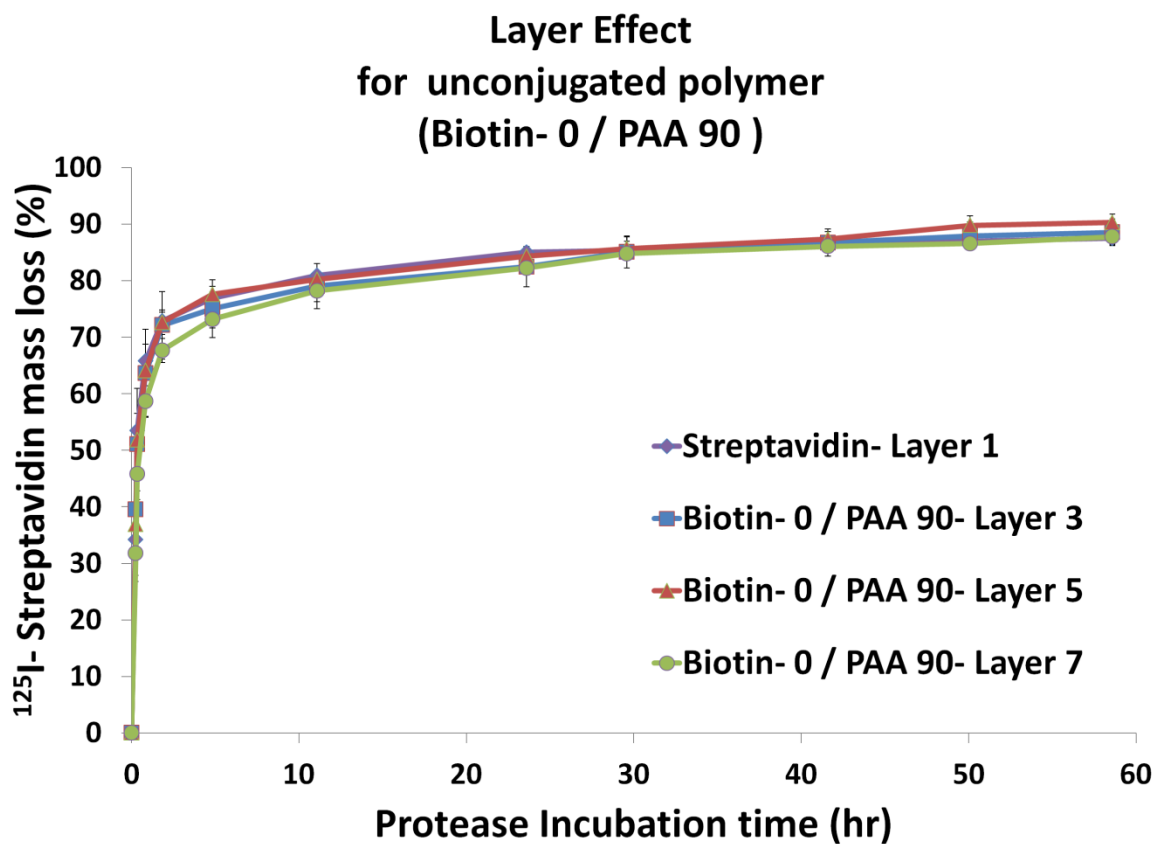


Figure A-4.1: LBL protease stability. Effect of factor “number of LBL layers (NoL)” from high MW (90,000 Da) unconjugated polymer (Biotin-0 / PAA 90), where an indistinguishable effect of number of assembly layers was noticed with increased assembly layers.

A.4.2. PAA (MW 90,000 Da) materials - layer effects in LBLs of Biotin-5.5/PAA 90

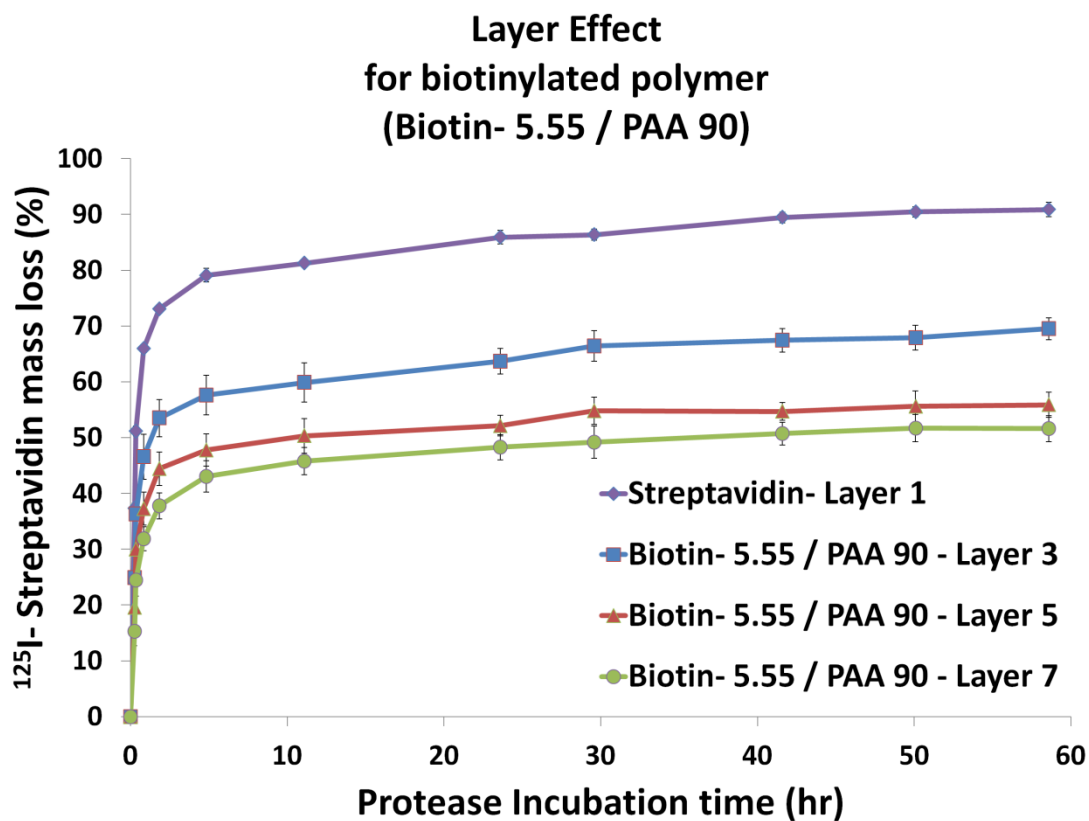


Figure A-4.2: LBL protease stability. Effect of factor “number of LBL layers (NoL)” from high MW (90,000 Da) biotinylated polymer (Biotin-5.55 / PAA 90) demonstrated significant distinguishable barrier properties, where increased assembly layers resulted in better chemical stability.

A.5. Protease stability on LBLs of midrange MW polymers studying conjugation effect

A.5.1. PAA (MW 50,000 Da) materials - conjugation effects in 3-layered assemblies

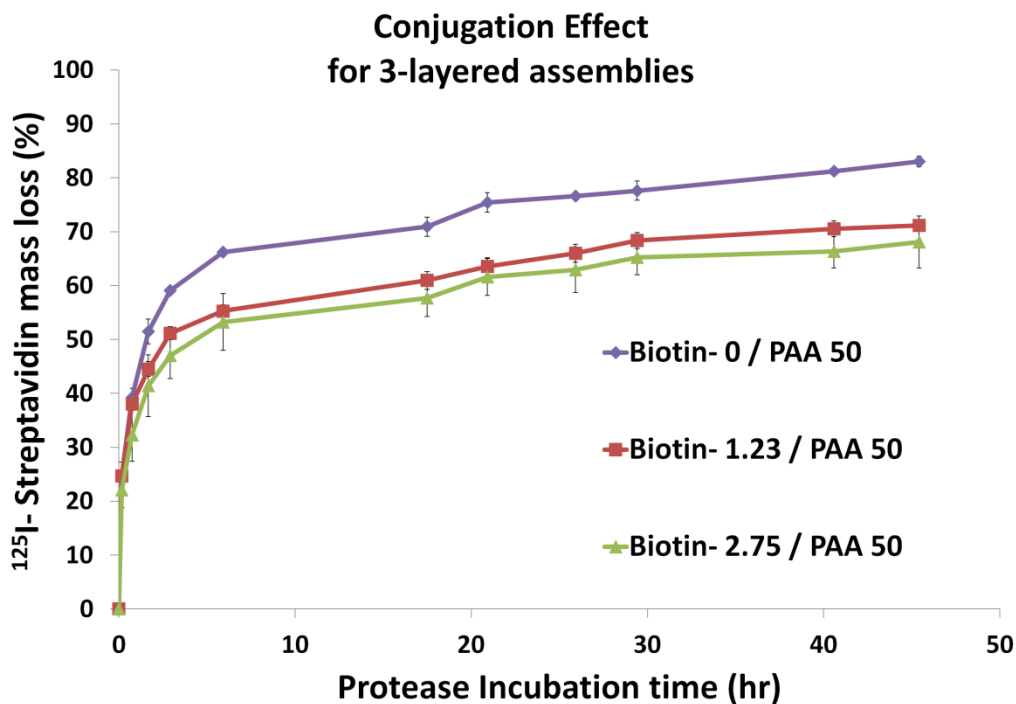


Figure A-5.1: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50) studying effect of factor “extent of biotin conjugation” from 3-layered assemblies (NoL=3) of varying extent of biotinylation (Biotin-0/1.23/2.75). From plots it was observed, biotinylated polymers (Biotin-1.23/2.75) were distinguishable from unconjugated polymers and showed improved chemical stability.

A.5.2. PAA (MW 50,000 Da) materials - conjugation effects in 5-layered assemblies

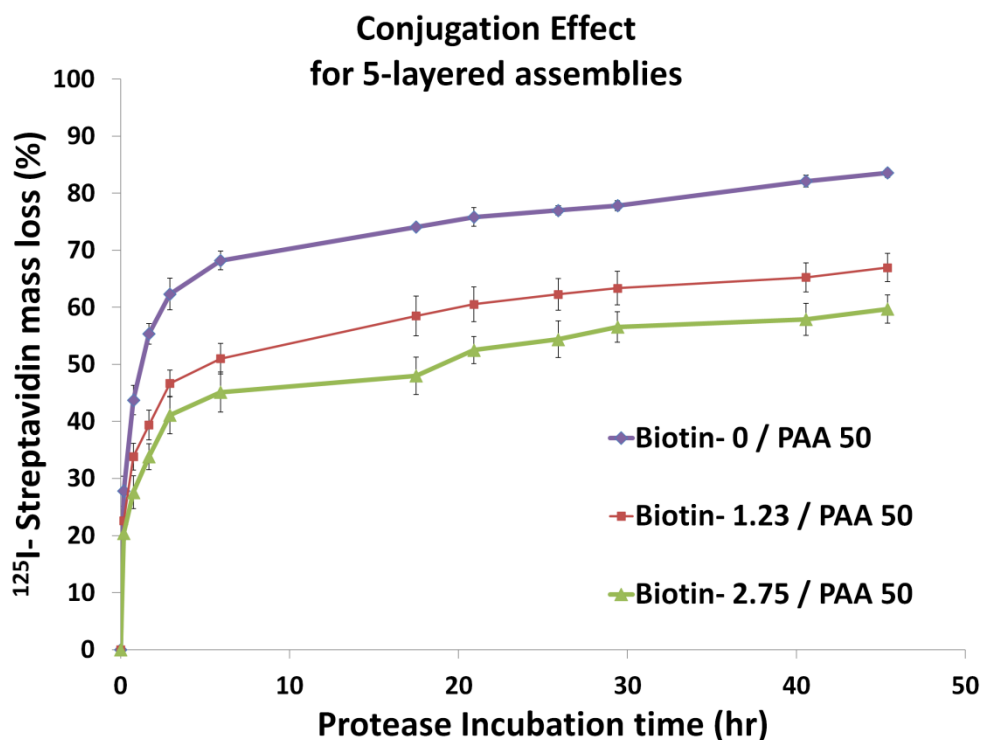


Figure A-5.2: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50) studying effect of factor “extent of biotin conjugation” from 5-layered assemblies (NoL=5) of varying extent of biotinylation (Biotin-0/1.23/2.75). From plots it was observed, biotinylated polymers (Biotin-1.23/2.75) were distinguishable from unconjugated polymers and showed improved chemical stability.

A.5.3. PAA (MW 50,000 Da) materials - conjugation effects in 7-layered assemblies

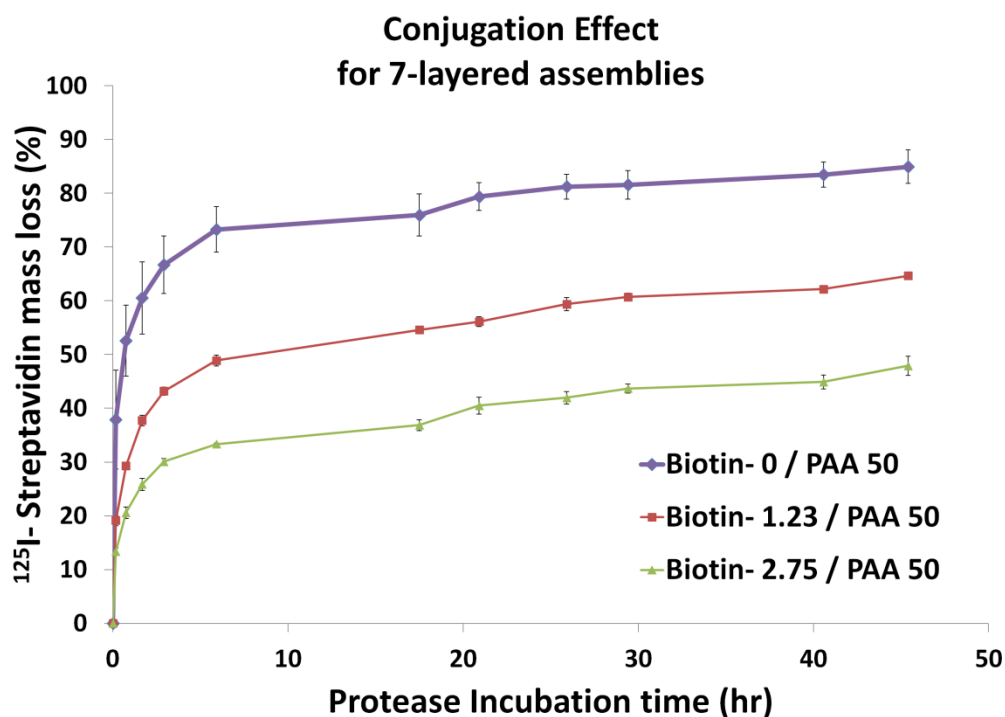


Figure A-5.3: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50) studying effect of factor “extent of biotin conjugation” from 7-layered assemblies (NoL=7) of varying extent of biotinylation (Biotin-0/1.23/2.75). From plots it was observed, biotinylated polymers (Biotin-1.23/2.75) were distinguishable from unconjugated polymers and showed improved chemical stability.

A.6. Protease stability on LBLs of midrange MW polymers studying layer effect

A.6.1. PAA (MW 50,000 Da) materials - layer effects in LBLs of unconjugated polymer

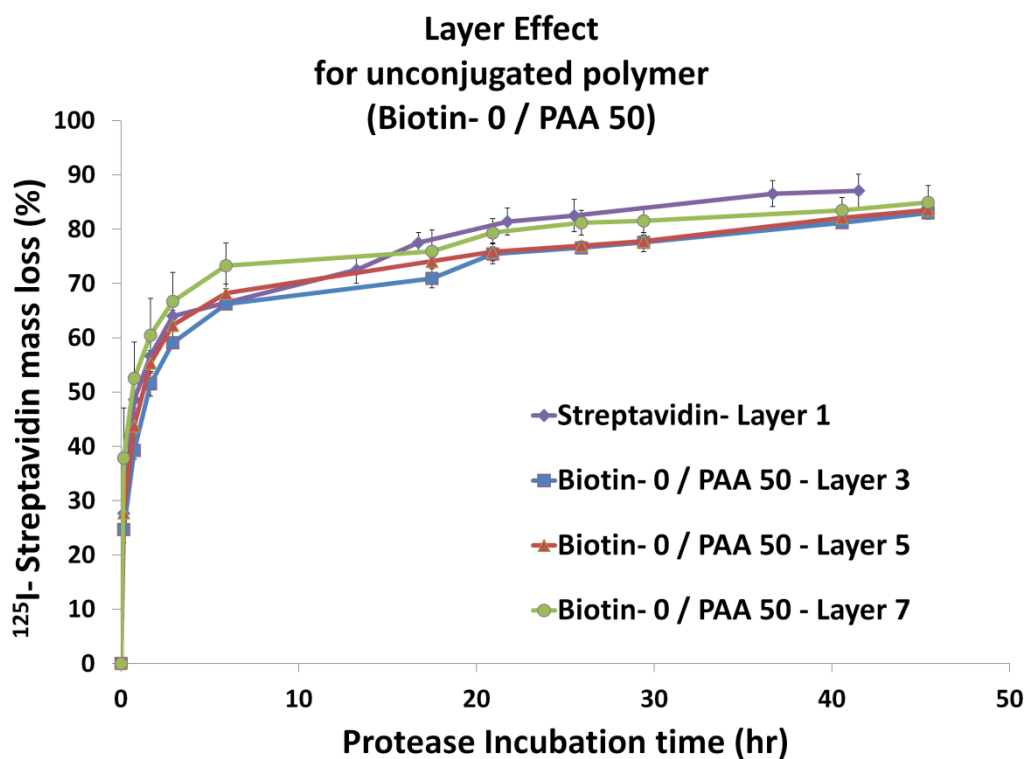


Figure A-6.1: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50 studying effect of factor “number of LBL layers (NoL)”. From LBLs of unconjugated polymer (Biotin-0 / PAA 50), it was observed the effects were indistinguishable with increased number of assembly layers.

A.6.2. PAA (MW 50,000 Da) materials - layer effects in LBLs of Biotin- 1.23 / PAA 50

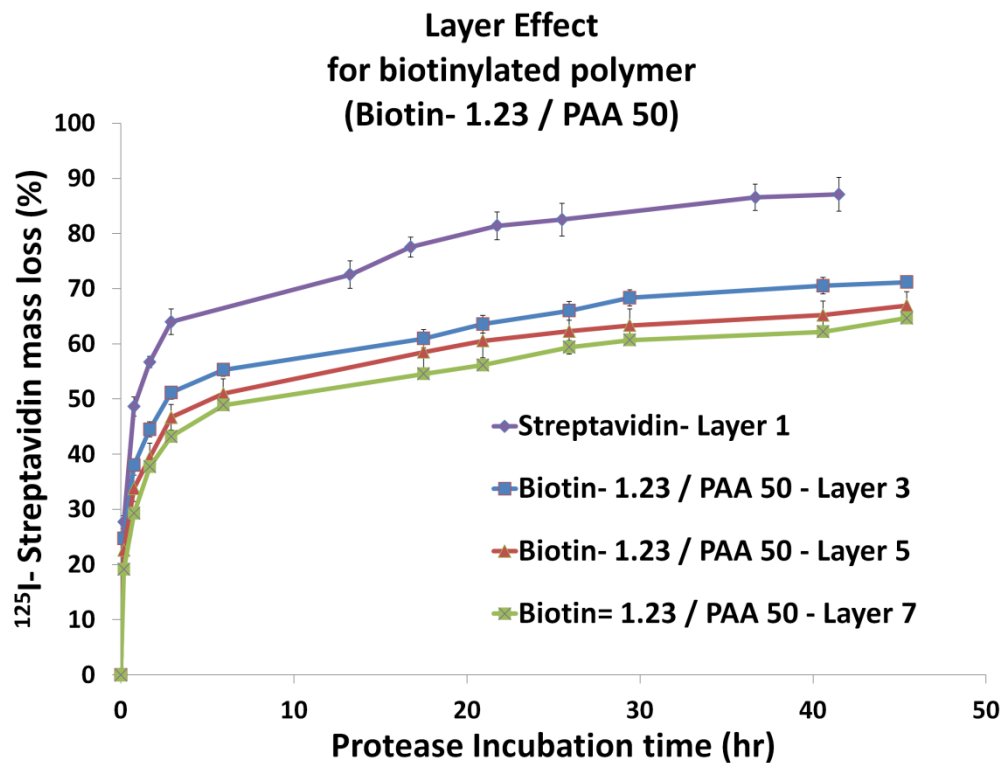


Figure A-6.2: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50 studying effect of factor “number of LBL layers (NoL)”. From LBLs of biotinylated polymer (Biotin-1.23 / PAA 50) a distinguishable effect with increased number of assembly layers was observed.

A.6.3. PAA (MW 50,000 Da) materials - layer effects in LBLs of Biotin- 2.75 / PAA 50

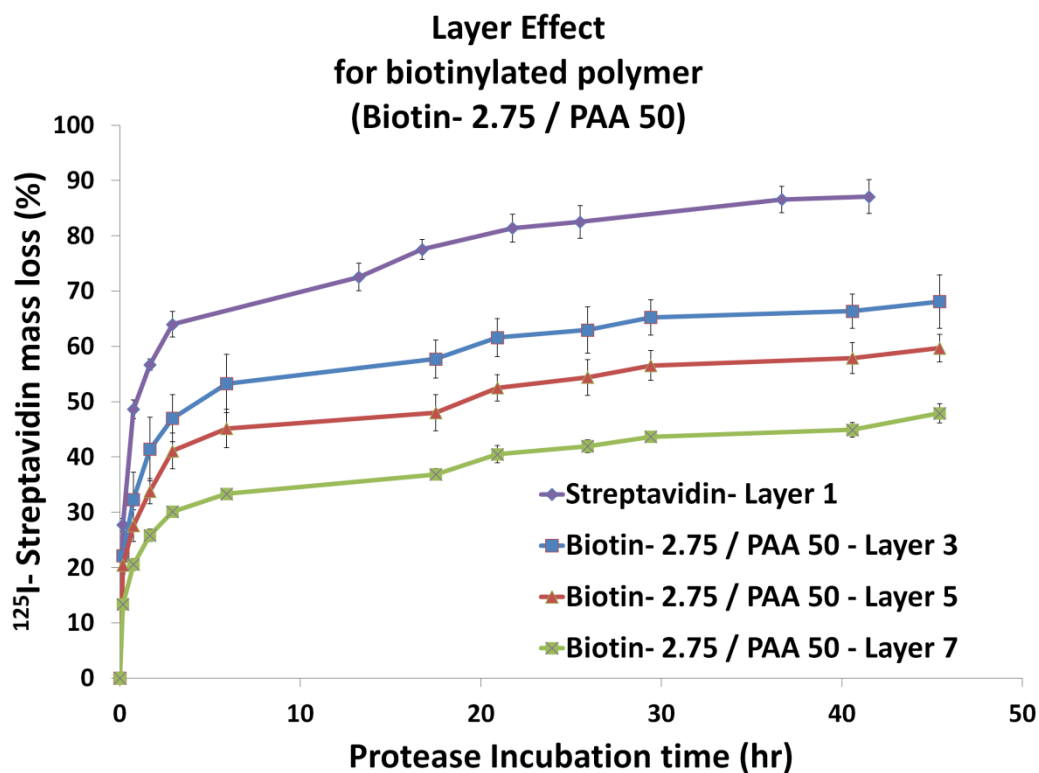


Figure A-6.3: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50) studying effect of factor “number of LBL layers (NoL)”. From LBLs of biotinylated polymer (Biotin-2.75 / PAA 50) a distinguishable effect with increased number of assembly layers was observed.

A.6.4. Loss % from protease stability on LBLs of midrange MW (50,000 Da) polymers

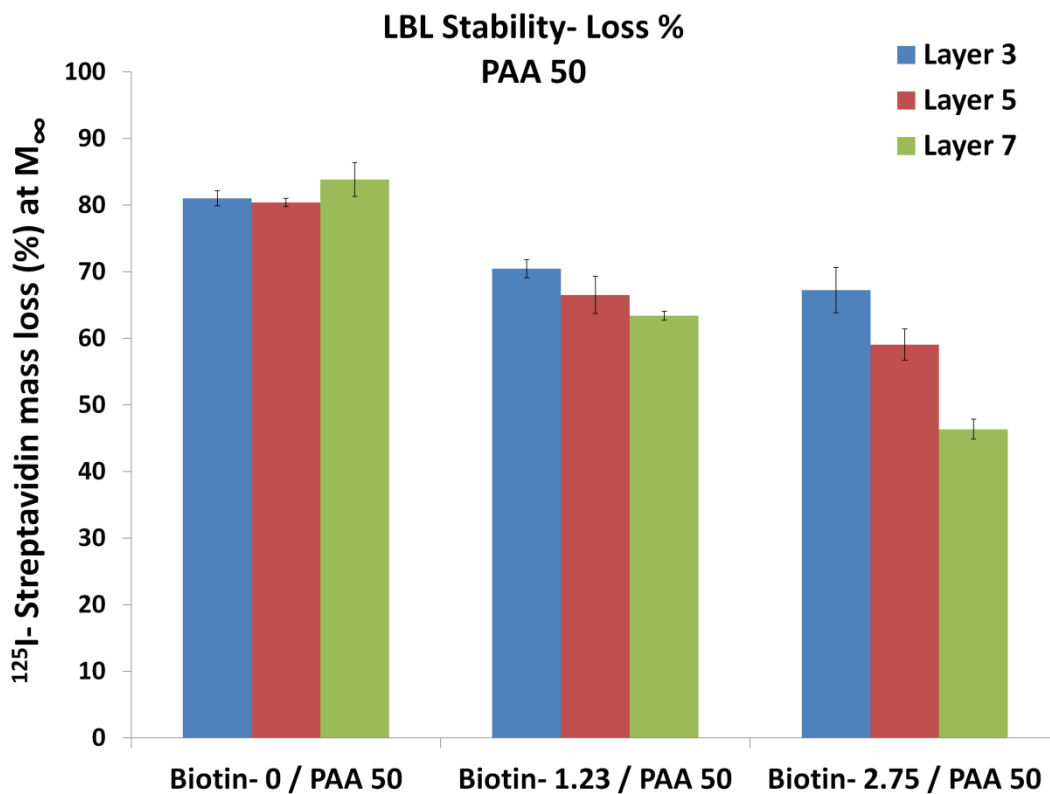


Figure A-6.4: Protease stability on LBLs developed of mid-range MW (50,000 Da) polymeric materials (PAA 50) studying loss % at M_{∞} for LBL assemblies developed of various materials. From plots it was observed, biotinylated polymers (Biotin-1.23/2.75) resulted in improved chemical stability with increased assembly layers.

A.7. Multivariate analysis on LBL protease stability (uncertainty plots)

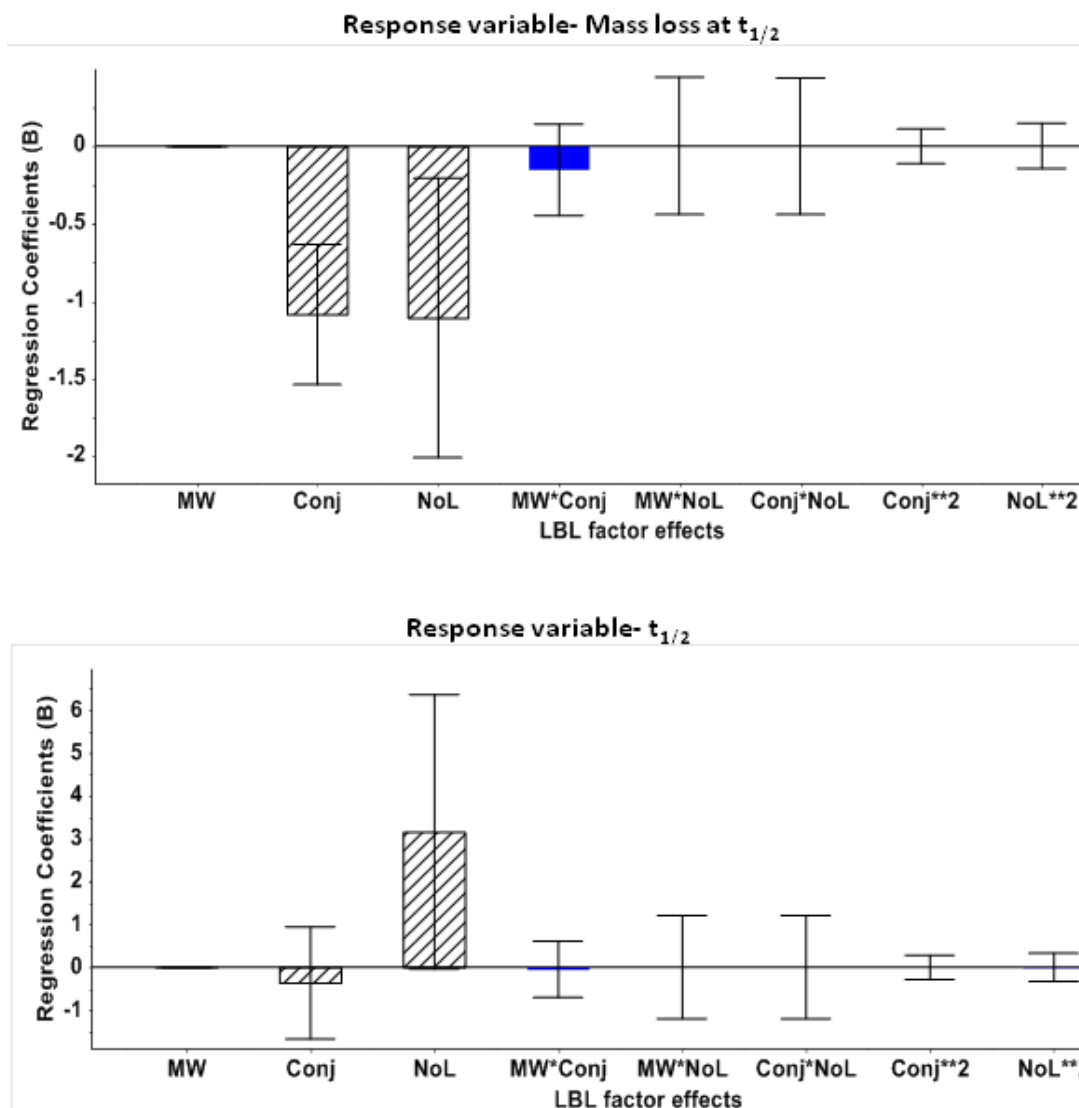


Figure A-7: PCR analysis on LBL protease stability studying LBL factor effects. Plot shows principal component regression (PCR) analysis on regression coefficient (B) for various LBL factors while considering variable (mass loss for $t_{1/2}$ and $t_{1/2}$) during analysis. Test of significance on regression coefficient (B) was performed using uncertainty tests. LBL factor effects with its uncertainty limit crossing over the zero axes were considered insignificant.

A.8. Salivary stability on LBLs of high MW polymers studying conjugation effect

A.8.1. PAA (MW 90,000 Da) materials - conjugation effect in 3-layered assemblies

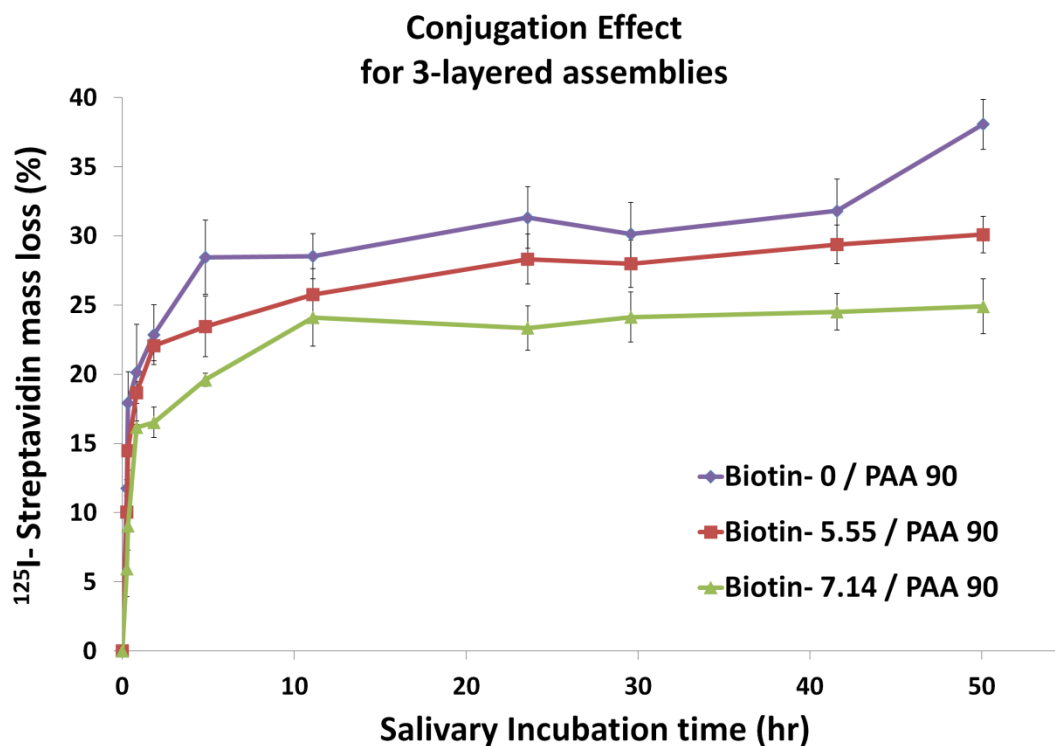


Figure A-8.1: LBL salivary stability. Effect of factor “extent of biotin conjugation” from 3-layered assemblies (NoL=3) developed of high MW (90,000 Da) polymer with varying extent of biotinylation (Biotin- 0/5.55/7.1).

A.8.2. PAA (MW 90,000 Da) materials - conjugation effect in 5-layered assemblies

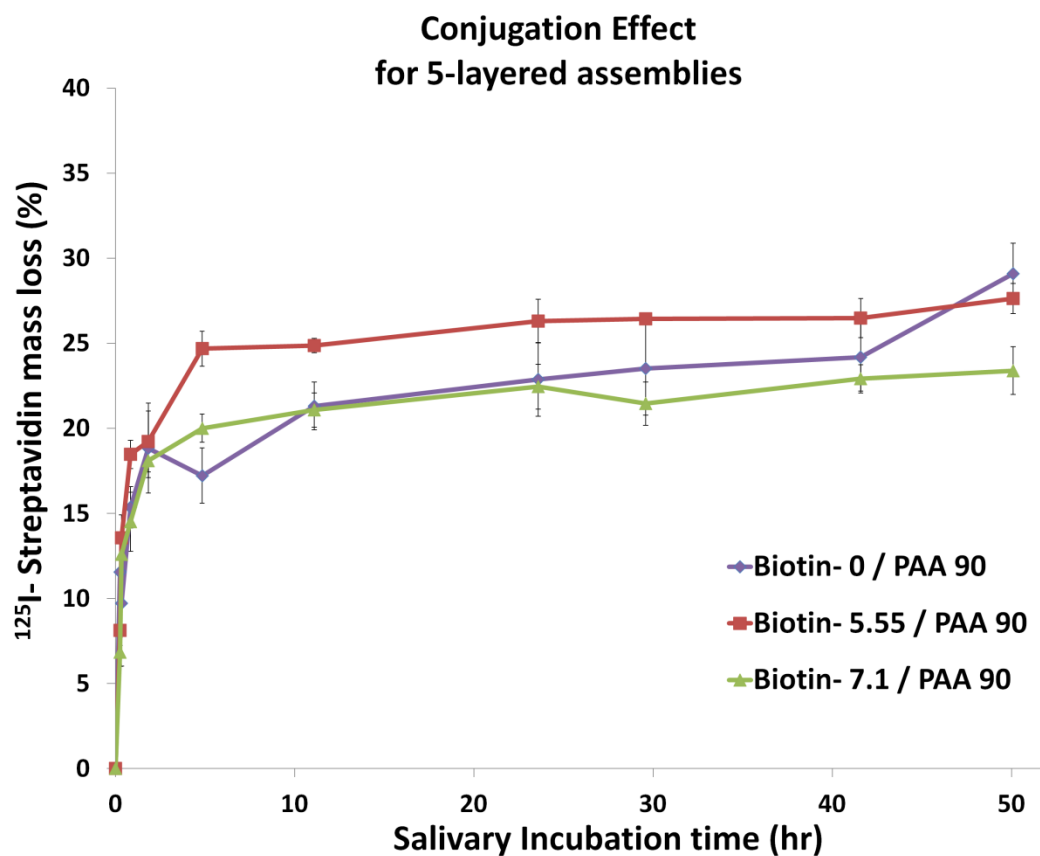


Figure A-8.2: LBL salivary stability. Effect of factor “extent of biotin conjugation” from 5-layered assemblies (NoL=5) developed of high MW (90,000 Da) polymer with varying extent of biotinylation (Biotin- 0/5.55/7.1).

A.9. Salivary stability on LBLs of high MW polymers studying layer effect

A.9.1. PAA (MW 90,000 Da) materials - layer effects in LBLs of unconjugated polymer

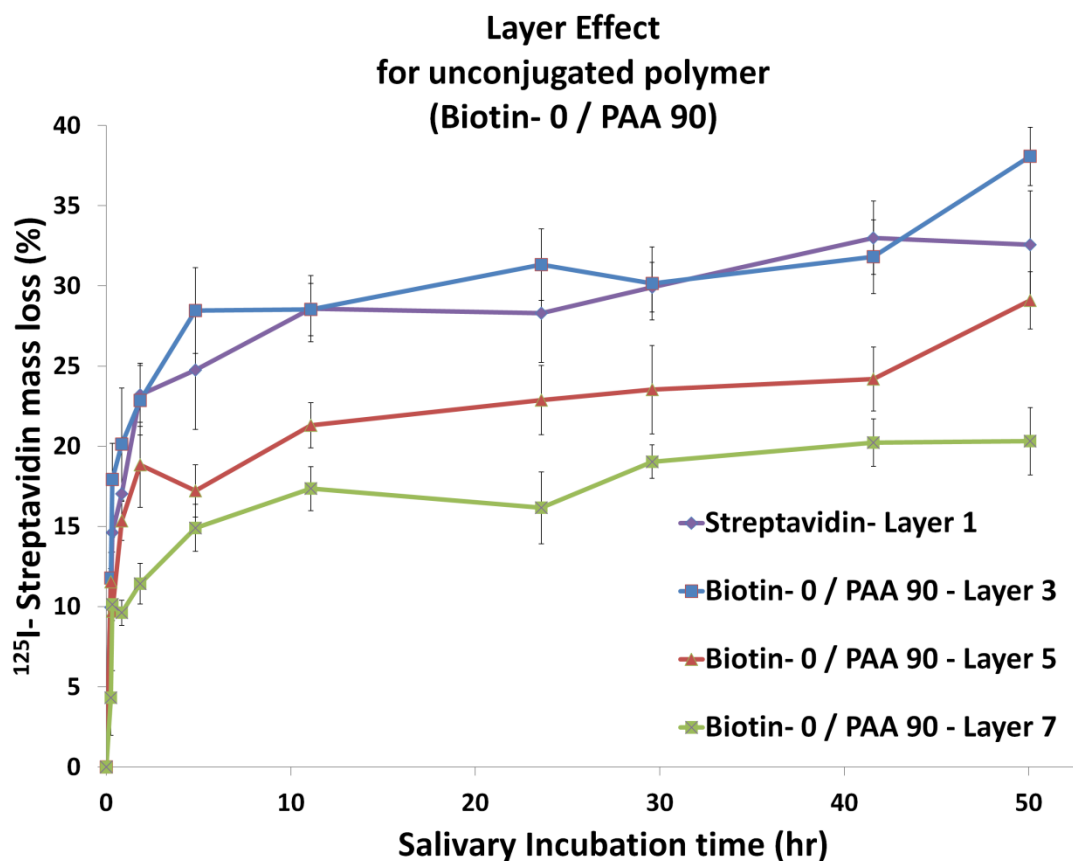


Figure A-9.1: LBL salivary stability. Effect of factor “number of LBL layers (NoL)” from high MW (90,000 Da) unconjugated polymer (Biotin-0 / PAA 90).

A.9.2. PAA (MW 90,000 Da) materials - layer effects in LBLs of Biotin- 5.55 / PAA 90

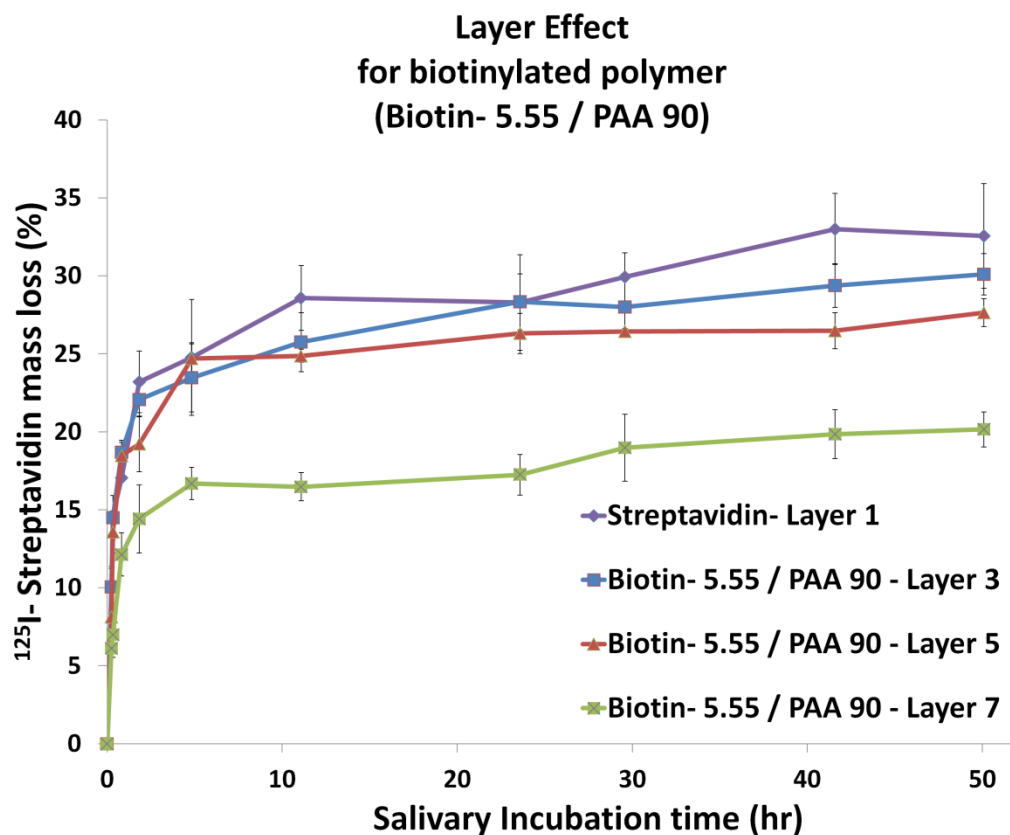


Figure A-9.2: LBL salivary stability. Effect of factor “number of LBL layers (NoL)” from high MW (90,000 Da) biotinylated polymer (Biotin-5.55 / PAA 90) demonstrated significant distinguishable barrier properties, where increased assembly layers resulted in better chemical stability.

A.10. Multivariate analysis on LBL salivary stability

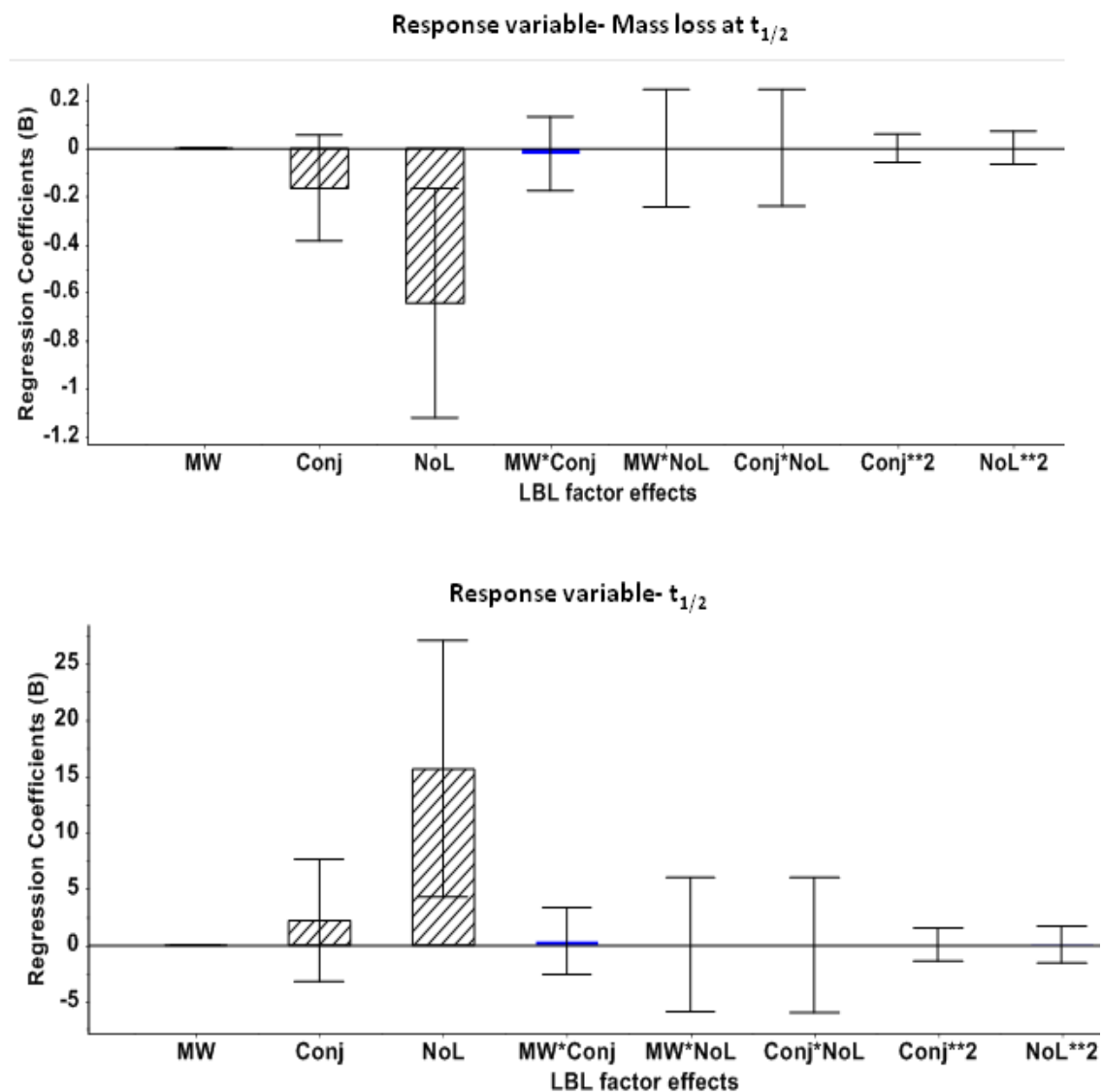


Figure A-10: PCR analysis on LBL factors (molecular weight, conjugation and number of layers). Plot shows principal component regression (PCR) analysis on regression coefficient (B) for various LBL factors while considering variable (mass loss for $t_{1/2}$ and $t_{1/2}$) during analysis. Test of significance on regression coefficient (B) was performed using uncertainty tests. LBL factor effects with its uncertainty limit crossing over the zero axes were considered insignificant.

A.11. Main effects plot for LBL chemical stability for response “Mass loss% for $t_{1/2}$ ”

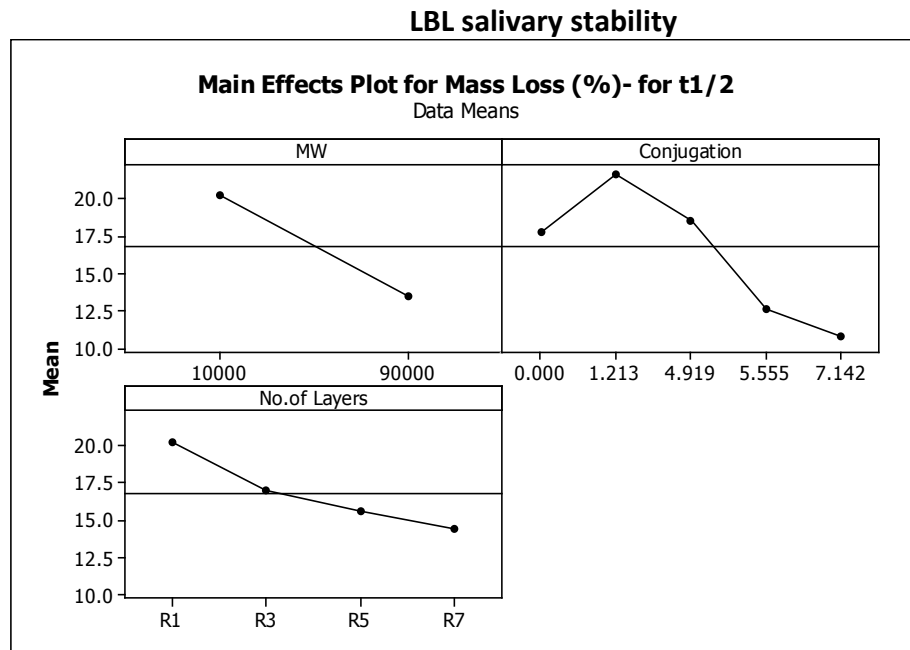
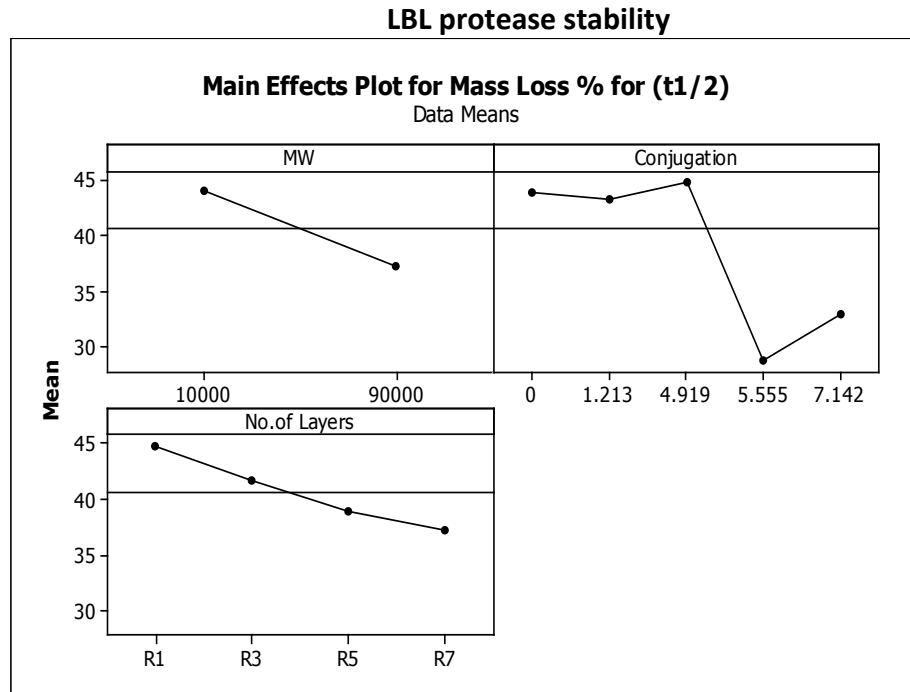


Figure A-11: LBL chemical stability. Plot shows main effect trends in LBL factors for response “Mass loss% for $t_{1/2}$ ” obtained from ANOVA plots demonstrates the trend involved.

LIST OF ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of Variance
AP	Activator protein
BMI	Body Mass Index
BSA	Bovine Serum Albumin
Conj	Conjugation (extent of biotinylation)
CREKA	Cys-Arg-Glu-Lys-Ala
CTC	Common Toxicity Criteria
COX	Cyclooxygenase
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDC	1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GA	glycrrhetic acid
GLM	Generalized Linear Model
HA	Hyaluronic acid
HABA	4'-hydroxyazobenzene-2-carboxylic acid
HNC	Head and Neck Cancer
HPC	hydroxypropyl-cellulose
HSCT	Hematopoietic Stem Cell Transplants
IL	Interleukin
ITF	Intestinal trefoil factor
Laser	Light amplification by stimulated emission of radiation
LBL	Layer-by-Layer
KGF	Keratinocyte growth factor
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MMP	Matrix metalloproteinases
MW	Molecular weight
NCI	National Cancer Institute
NF- κ B	Nuclear factor- κ B
NHS	<i>N</i> -hydroxysuccinimide
NoL	Number of layers
MW	Molecular weight
OM	Oral mucositis
OMAS	Oral mucositis Assessment Scale
PAA	Poly(acrylic acid)
p53	tumor protein 53
PBS	Phosphate-buffered saline
PC	Principal Component

PCA	Principal Component Analysis
PCR	Principal Component Regression
PEM	Polyelectrolyte Multilayers
PVP	polyvinyl pyrrolidone
rh	recombinant human
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase- High Performance Liquid Chromatography
Sulfo-NHS	N-hydroxysulfosuccinimide
STAT3	Signal transducer and activator of transcription 3
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TNF	Tumor-necrosis factor
UF	Ultrafiltration
UWS	Unstimulated Whole Saliva
VEGF	Vascular endothelial growth factor
WCCNR	Western Consortium for Cancer Nursing Research
WHO	World Health Organization
WoS	Work of Separation

REFERENCES

1. Saadeh, C.E., *Chemotherapy- and radiotherapy-induced oral mucositis: Review of preventive strategies and treatment*. Pharmacotherapy, 2005. **25**(4): p. 540-554.
2. Duncan, M. and G. Grant, *Review article: oral and intestinal mucositis - causes and possible treatments*. Alimentary Pharmacology & Therapeutics, 2003. **18**(9): p. 853-874.
3. Karthaus, M., C. Rosenthal, and A. Ganser, *Prophylaxis and treatment of chemo- and radiotherapy-induced oral mucositis - are there new strategies?* Bone Marrow Transplantation, 1999. **24**(10): p. 1095-1108.
4. Sonis, S.T., *Oral mucositis*. Anti-Cancer Drugs, 2011. **22**(7): p. 607-612.
5. Farrington, M. and L. Cullen, *Feature: Oral mucositis- Oral care considerations during patient's cancer treatment*, in *Oncology Nurse Advisor*. 2010. p. 24-28.
6. Epstein, J.B. and M.M. Schubert, *Managing pain in mucositis*. Semin Oncol Nurs, 2004. **20**(1): p. 30-7.
7. Peterson, D.E., et al., *Management of oral and gastrointestinal mucositis: ESMO Clinical Recommendations*. Annals of Oncology, 2009. **20**: p. 174-177.
8. Sonis, S.T., *Mucositis: The impact, biology and therapeutic opportunities of oral mucositis*. Oral Oncology, 2009. **45**(12): p. 1015-1020.
9. Nonzee, N.J., et al., *Evaluating the Supportive Care Costs of Severe Radiochemotherapy-Induced Mucositis and Pharyngitis: Results From a Northwestern University Costs of Cancer Program Pilot Study With Head and Neck and Non-small Cell Lung Cancer Patients Who Received Care at a County Hospital, a Veterans Administration Hospital, or a Comprehensive Cancer Care Center (vol 113, pg 1446, 2008)*. Cancer, 2009. **115**(12): p. 2805-2805.
10. Mueller, B.A., et al., *Mucositis management practices for hospitalized patients: national survey results*. J Pain Symptom Manage, 1995. **10**(7): p. 510-20.
11. Coates, A., et al., *On the Receiving End Patient Perception of the Side-Effects of Cancer-Chemotherapy*. European Journal of Cancer & Clinical Oncology, 1983. **19**(2): p. 203-208.

12. Trotti, A., et al., *Common toxicity criteria: Version 2.0. an improved reference for grading the acute effects of cancer treatment: Impact on radiotherapy*. International Journal of Radiation Oncology Biology Physics, 2000. **47**(1): p. 13-47.
13. Scully, C., J. Epstein, and S. Sonis, *Oral mucositis: A challenging complication of radiotherapy, chemotherapy, and radiochemotherapy: Part 1, pathogenesis and prophylaxis of mucositis*. Head and Neck-Journal for the Sciences and Specialties of the Head and Neck, 2003. **25**(12): p. 1057-1070.
14. Stiff, P., *Mucositis associated with stem cell transplantation: current status and innovative approaches to management*. Bone Marrow Transplant, 2001. **27 Suppl 2**: p. S3-S11.
15. Brown, C.G. and J. Wingard, *Clinical consequences of oral mucositis*. Semin Oncol Nurs, 2004. **20**(1): p. 16-21.
16. Raber-Durlacher, J.E., S. Elad, and A. Barasch, *Oral mucositis*. Oral Oncology, 2010. **46**(6): p. 452-456.
17. Cawley, M.M. and L.M. Benson, *Current trends in managing oral mucositis*. Clin J Oncol Nurs, 2005. **9**(5): p. 584-92.
18. Worthington, H.V., J.E. Clarkson, and O.B. Eden, *Interventions for preventing oral mucositis for patients with cancer receiving treatment*. Cochrane Database of Systematic Reviews, 2006(2).
19. Bellm, L.A., et al., *Patient reports of complications of bone marrow transplantation*. Supportive Care in Cancer, 2000. **8**(1): p. 33-9.
20. Sonis, S.T., *Mucositis as a biological process: a new hypothesis for the development of chemotherapy-induced stomatotoxicity*. Oral Oncology, 1998. **34**(1): p. 39-43.
21. Sonis, S.T., et al., *Oral mucositis and the clinical and economic outcomes of hematopoietic stem-cell transplantation*. Journal of Clinical Oncology, 2001. **19**(8): p. 2201-2205.
22. Donnelly, J.P., et al., *Antimicrobial therapy to prevent or treat oral mucositis*. Lancet Infectious Diseases, 2003. **3**(7): p. 405-412.
23. Society, A.C. American Cancer Society. *Cancer Facts & Figures 2011*. 2011; Available from:

<http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-029771.pdf>.

24. Barasch, A. and D.E. Peterson, *Risk factors for ulcerative oral mucositis in cancer patients: unanswered questions*. Oral Oncology, 2003. **39**(2): p. 91-100.
25. Raber-Durlacher, J.E., et al., *Oral mucositis in patients treated with chemotherapy for solid tumors: a retrospective analysis of 150 cases*. Supportive Care in Cancer, 2000. **8**(5): p. 366-71.
26. McGuire, D.B., et al., *Patterns of mucositis and pain in patients receiving preparative chemotherapy and bone marrow transplantation*. Oncol Nurs Forum, 1993. **20**(10): p. 1493-502.
27. Woo, S.B., et al., *A longitudinal study of oral ulcerative mucositis in bone marrow transplant recipients*. Cancer, 1993. **72**(5): p. 1612-7.
28. Kostler, W.J., et al., *Oral mucositis complicating chemotherapy and/or radiotherapy: options for prevention and treatment*. CA Cancer J Clin, 2001. **51**(5): p. 290-315.
29. Rugg, T., M.I. Saunders, and S. Dische, *Smoking and mucosal reactions to radiotherapy*. Br J Radiol, 1990. **63**(751): p. 554-6.
30. Verdi, C.J., *Cancer therapy and oral mucositis. An appraisal of drug prophylaxis*. Drug Saf, 1993. **9**(3): p. 185-95.
31. Sonis, S.T. and E.G. Fey, *Oral complications of cancer therapy*. Oncology-New York, 2002. **16**(5): p. 680-686.
32. Cengiz, M., et al., *Sucralfate in the prevention of radiation-induced oral mucositis*. J Clin Gastroenterol, 1999. **28**(1): p. 40-3.
33. Shih, A., et al., *Mechanisms for radiation-induced oral mucositis and the consequences*. Cancer Nurs, 2003. **26**(3): p. 222-9.
34. Backstrom, I., et al., *Dietary intake in head and neck irradiated patients with permanent dry mouth symptoms*. Eur J Cancer B Oral Oncol, 1995. **31B**(4): p. 253-7.
35. Madeya, M.L., *Oral complications from cancer therapy: Part 2--Nursing implications for assessment and treatment*. Oncol Nurs Forum, 1996. **23**(5): p. 808-19.

36. Hickey, A.J., B.B. Toth, and S.B. Lindquist, *Effect of intravenous hyperalimentation and oral care on the development of oral stomatitis during cancer chemotherapy*. J Prosthet Dent, 1982. **47**(2): p. 188-93.
37. Sonis, S.T., *The pathobiology of mucositis*. Nat Rev Cancer, 2004. **4**(4): p. 277-84.
38. Sonis, S.T., *The biologic role for nuclear factor-kappaB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy*. Crit Rev Oral Biol Med, 2002. **13**(5): p. 380-9.
39. Sonis, S.T., *Pathobiology of oral mucositis: novel insights and opportunities*. J Support Oncol, 2007. **5**(9 Suppl 4): p. 3-11.
40. Hwang, D., et al., *Effects of ceramide inhibition on experimental radiation-induced oral mucositis*. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 2005. **100**(3): p. 321-329.
41. Bowen, J.M. and D.M. Keefe, *New pathways for alimentary mucositis*. J Oncol, 2008. **2008**: p. 907892.
42. Bamba, S., et al., *Matrix metalloproteinase-3 secretion from human colonic subepithelial myofibroblasts: role of interleukin-17*. Journal of Gastroenterology, 2003. **38**(6): p. 548-54.
43. Al-Dasooqi, N., et al., *Matrix metalloproteinases: key regulators in the pathogenesis of chemotherapy-induced mucositis?* Cancer Chemother Pharmacol, 2009. **64**(1): p. 1-9.
44. Sonis, S., et al., *Gene expression changes in peripheral blood cells provide insight into the biological mechanisms associated with regimen-related toxicities in patients being treated for head and neck cancers*. Oral Oncology, 2007. **43**(3): p. 289-300.
45. Blijlevens, N.M., J.P. Donnelly, and B.E. De Pauw, *Mucosal barrier injury: biology, pathology, clinical counterparts and consequences of intensive treatment for haematological malignancy: an overview*. Bone Marrow Transplant, 2000. **25**(12): p. 1269-78.
46. Ruescher, T.J., et al., *The impact of mucositis on alpha-hemolytic streptococcal infection in patients undergoing autologous bone marrow transplantation for hematologic malignancies*. Cancer, 1998. **82**(11): p. 2275-81.
47. Epstein, J.B. and M.M. Schubert, *Oropharyngeal mucositis in cancer therapy. Review of pathogenesis, diagnosis, and management*. Oncology (Williston Park), 2003. **17**(12): p. 1767-79; discussion 1779-82, 1791-2.

48. Al-Dasooqi, N., et al., *Irinotecan-induced alterations in intestinal cell kinetics and extracellular matrix component expression in the dark agouti rat*. Int J Exp Pathol, 2011. **92**(5): p. 357-365.
49. Moss, S.F. and N.A. Wright, *Molecular aspects of mucosal repair: a summary*. Yale J Biol Med, 1996. **69**(2): p. 155-8.
50. Hsiao, G., and Sonis, S., *Oral Mucositis*, in *Interactive Textbook on Clinical Symptom Research: Methods and Opportunities.*, B.M. Max, and Lynn, J., Editor. p. 8-10.
51. Jaroneski, L.A., *The importance of assessment rating scales for chemotherapy-induced oral mucositis*. Oncol Nurs Forum, 2006. **33**(6): p. 1085-90; quiz 1091-3.
52. Joshi, A. and P. Sloan, *Role of "fibrin" cuffs in chronic nonspecific oral ulceration*. Wound Repair and Regeneration, 2004. **12**(1): p. 18-23.
53. Scully, C., et al., *Oral lesions indicative of plasminogen deficiency (hypoplasminogenemia)*. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics, 2001. **91**(3): p. 334-337.
54. Ruoslahti, E., et al., *Targeting atherosclerosis by using modular, multifunctional micelles*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(24): p. 9815-9819.
55. Medly, J., et al., *Novel Fibrin Targeted Block Copolymers for the Prevention of Abdominal Postsurgical Adhesions*. Gastroenterology, 2011. **140**(5): p. S445-S445.
56. Ruoslahti, E., et al., *Targeting of albumin-embedded paclitaxel nanoparticles to tumors*. Nanomedicine-Nanotechnology Biology and Medicine, 2009. **5**(1): p. 73-82.
57. Dziubla, T.D., J.M. Medley, and J. Heisterberg, *Synthesis and Characterization of CREKA-Targeted Polymers for the Disruption of Fibrin Gel Matrix Propagation*. Journal of Biomaterials Science-Polymer Edition, 2011. **22**(10): p. 1363-1378.
58. Agemy, L., et al., *Nanoparticle-induced vascular blockade in human prostate cancer*. Blood, 2010. **116**(15): p. 2847-56.
59. Roopashri, G., Jayanthi, K., *Radiotherapy and Chemotherapy induced Oral Mucositis - Prevention and Current Therapeutic Modalities*. Indian journal of Dental Advancements, 2010. **2**(2): p. 174-179.

60. Sankar, V., et al., *Local drug delivery for oral mucosal diseases: challenges and opportunities*. Oral Diseases, 2011. **17 Suppl 1**: p. 73-84.
61. Sudhakar, Y., K. Kuotsu, and A.K. Bandyopadhyay, *Buccal bioadhesive drug delivery--a promising option for orally less efficient drugs*. Journal of Controlled Release, 2006. **114**(1): p. 15-40.
62. Plevova, P., *Prevention and treatment of chemotherapy- and radiotherapy-induced oral mucositis: a review*. Oral Oncology, 1999. **35**(5): p. 453-470.
63. Hearnden, V., et al., *New developments and opportunities in oral mucosal drug delivery for local and systemic disease*. Adv Drug Deliv Rev, 2011.
64. Pettit, L., et al., *Use of Mugard (TM) and Caphoso(R) in Patients with Locally Advanced Squamous Cell Carcinoma of the Head and Neck*. Clinical Oncology, 2011. **23**(3): p. S48-S48.
65. Buchsel, P.C., *Polyvinylpyrrolidone-sodium hyaluronate gel (Gelclair): a bioadherent oral gel for the treatment of oral mucositis and other painful oral lesions*. Expert Opin Drug Metab Toxicol, 2008. **4**(11): p. 1449-54.
66. Peterson, D.E., et al., *Phase II, randomized, double-blind, placebo-controlled study of recombinant human intestinal trefoil factor oral spray for prevention of oral mucositis in patients with colorectal cancer who are receiving fluorouracil-based chemotherapy*. Journal of Clinical Oncology, 2009. **27**(26): p. 4333-8.
67. Ryu, S.H., et al., *Therapeutic Effects of Recombinant Human Epidermal Growth Factor (rhEGF) in a Murine Model of Concurrent Chemo- and Radiotherapy-Induced Oral Mucositis*. Journal of Radiation Research, 2010. **51**(5): p. 595-601.
68. Stokman, M.A., et al., *Clinical effects of flurbiprofen tooth patch on radiation-induced oral mucositis. A pilot study*. Supportive Care in Cancer, 2005. **13**(1): p. 42-8.
69. (2007) *Magic Mouthwash* PHARMACIST'S LETTER / PRESCRIBER'S LETTER **23**.
70. Chan, A. and R.J. Ignoffo, *Survey of topical oral solutions for the treatment of chemo-induced oral mucositis*. J Oncol Pharm Pract, 2005. **11**(4): p. 139-43.
71. Rubenstein, E.B., et al., *Clinical practice guidelines for the prevention and treatment of cancer therapy-induced oral and gastrointestinal mucositis*. Cancer, 2004. **100**(9): p. 2026-2046.

72. Needleman, I.G., F.C. Smales, and G.P. Martin, *An investigation of bioadhesion for periodontal and oral mucosal drug delivery*. Journal of Clinical Periodontology, 1997. **24**(6): p. 394-400.
73. Oguchi, M., et al., *Mucosa-adhesive water-soluble polymer film for treatment of acute radiation-induced oral mucositis*. Int J Radiat Oncol Biol Phys, 1998. **40**(5): p. 1033-7.
74. Yamamura, K., et al., *Oral mucosal adhesive film containing local anesthetics: In vitro and clinical evaluation*. Journal of Biomedical Materials Research, 1998. **43**(3): p. 313-317.
75. World Health Organization. *Handbook for reporting the results of cancer treatment*. . 1979: p. 15-22.
76. DCTD, N., NIH, DHHS *Cancer Therapy Evaluation Program: Common Toxicity Criteria, Version 2.0*.
77. Sonis, S.T., et al., *Validation of a new scoring system for the assessment of clinical trial research of oral mucositis induced by radiation or chemotherapy*. Mucositis Study Group. Cancer, 1999. **85**(10): p. 2103-13.
78. *Development of a staging system for chemotherapy-induced stomatitis*. Western Consortium for Cancer Nursing Research. Cancer Nurs, 1991. **14**(1): p. 6-12.
79. Karagozoglu, S. and M. Filiz Ulusoy, *Chemotherapy: the effect of oral cryotherapy on the development of mucositis*. J Clin Nurs, 2005. **14**(6): p. 754-65.
80. Baydar, M., et al., *Prevention of oral mucositis due to 5-fluorouracil treatment with oral cryotherapy*. J Natl Med Assoc, 2005. **97**(8): p. 1161-4.
81. Peterson, D.E., *Oral and Gastrointestinal mucositis: novel insights into pathophysiology and potential therapies* Advanced Studies in Medicine, 2005. **5**(4(B)): p. S299-S310.
82. Lopes, N.N.F., et al., *Cyclooxygenase-2 and vascular endothelial growth factor expression in 5-fluorouracil-induced oral mucositis in hamsters: evaluation of two low-intensity laser protocols*. Supportive Care in Cancer, 2009. **17**(11): p. 1409-1415.
83. Paesmans, M., et al., *The use of low-energy laser (LEL) for the prevention of chemotherapy- and/or radiotherapy-induced oral mucositis in cancer patients: results from two prospective studies*. Supportive Care in Cancer, 2008. **16**(12): p. 1381-1387.

84. Epstein, J.B., et al., *Benzydamine HCl for prophylaxis of radiation-induced oral mucositis - Results from a multicenter, randomized, double-blind, placebo-controlled clinical trial*. Cancer, 2001. **92**(4): p. 875-885.
85. Hanna, E.Y., *Prevention of radiation-induced oral mucositis with benzydamine*. Curr Oncol Rep, 2002. **4**(1): p. 65-6.
86. Elad, S., et al., *Systemic absorption of lidocaine after topical application for the treatment of oral mucositis in bone marrow transplantation patients*. J Oral Pathol Med, 1999. **28**(4): p. 170-2.
87. Young, J.L., Bogner, R.H., *Case Report: Lidocaine Mucoadhesive Buccal Tablets for Local relief for Mouth Ulcers*. International Journal of Pharmaceutical Compounding 2009. **13**(3): p. 214-217.
88. Epstein, J.B., et al., *Assessment of epidermal growth factor in oral secretions of patients receiving radiation therapy for cancer*. Oral Oncology, 1997. **33**(5): p. 359-63.
89. Sonis, S.T., et al., *Prevention of chemotherapy-induced ulcerative mucositis by transforming growth factor beta 3*. Cancer Research, 1994. **54**(5): p. 1135-8.
90. Krijanovski, O.I., et al., *Keratinocyte growth factor separates graft-versus-leukemia effects from graft-versus-host disease*. Blood, 1999. **94**(2): p. 825-31.
91. Blijlevens, N. and S. Sonis, *Palifermin (recombinant keratinocyte growth factor-1): a pleiotropic growth factor with multiple biological activities in preventing chemotherapy- and radiotherapy-induced mucositis*. Annals of Oncology, 2007. **18**(5): p. 817-26.
92. Sonis, S.T., *A biological approach to mucositis*. J Support Oncol, 2004. **2**(1): p. 21-32; discussion 35-6.
93. Sonis, S.T., *Efficacy of palifermin (keratinocyte growth factor-1) in the amelioration of oral mucositis*. Core Evid, 2010. **4**: p. 199-205.
94. Epstein, J.B., et al., *The correlation between epidermal growth factor levels in saliva and the severity of oral mucositis during oropharyngeal radiation therapy*. Cancer, 2000. **89**(11): p. 2258-2265.
95. Hong, J.P., et al., *Recombinant human epidermal growth factor treatment of radiation-induced severe oral mucositis in patients with head and neck malignancies*. Eur J Cancer Care (Engl), 2009. **18**(6): p. 636-41.

96. Ryu, S.H., et al., *Therapeutic effects of recombinant human epidermal growth factor (rhEGF) in a murine model of concurrent chemo- and radiotherapy-induced oral mucositis*. J Radiat Res (Tokyo), 2010. **51**(5): p. 595-601.
97. Stenger, M.a.P., A., *Calcium phosphate mouth rinse for preventing oral mucositis: A new artificial saliva protects the mucosa from damage due to cancer treatment*. Community Oncology, 2008. **5**(4): p. 171-172.
98. Papas, A., *Caphosol reduces the severity of mucositis when started at the onset of cancer therapy* Community Oncology, 2008. **5**(4): p. 172-173.
99. Price, A., *From the Nurse's Perspective: Mucositis often overlooked and undertreated*. Community Oncology, 2008. **5**(4): p. 173-174.
100. U.S. Food and Drug Administration, D.o.H.a.H.S. *Summary of Safety and Effectiveness: Caphosol Artificial Saliva, Inpharma, AS*. 2003; Available from: http://www.accessdata.fda.gov/cdrh_docs/pdf3/k030802.pdf.
101. Swaminathan, D. and S. Uma, *The efficacy of an alcohol-free 0.12 % w/v chlorhexidine gluconate mouthwash (Oradex (R))*. Journal of Dental Research, 2001. **80**(4): p. 1385-1385.
102. AL-Bayat, F.H., et al., *Antibacterial effects of Oradex, Gengigel and Salviathymol-n mouthwash on dental biofilm bacteria*. African Journal of Microbiology Research, 2011. **5**(6): p. 636-642.
103. U.S. Food and Drug Administration, D.o.H.a.H.S. *510(k) for Gelclair Concentrated Oral Gel*. 2001; Available from: http://www.accessdata.fda.gov/cdrh_docs/pdf/k013056.pdf.
104. *Gelclair Product Information*. Available from: <http://www.gelclair.net/gpi.html>.
105. Innocenti, M., G. Moscatelli, and S. Lopez, *Efficacy of gelclair in reducing pain in palliative care patients with oral lesions: preliminary findings from an open pilot study*. J Pain Symptom Manage, 2002. **24**(5): p. 456-7.
106. Barber, C., et al., *Comparing pain control and ability to eat and drink with standard therapy vs Gelclair: a preliminary, double centre, randomised controlled trial on patients with radiotherapy-induced oral mucositis*. Supportive Care in Cancer, 2007. **15**(4): p. 427-40.
107. *Carrington Laboratories, Inc. (CARN) and Primus Pharmaceuticals, Inc. Sign Long-Term Supply and Patent Licensing Agreements for Acemannan Hydrogel(TM)*.

2007; Available from: <http://www.biospace.com/News/carrington-laboratories-inc-and-primus/52880>.

108. Thomas, D.R., et al., *Acemannan hydrogel dressing versus saline dressing for pressure ulcers. A randomized, controlled trial*. Adv Wound Care, 1998. **11**(6): p. 273-6.
109. Bhat, G., Dodwad, V., Kudva, P., *Aloe vera: Nature's soothing healer to periodontal disease*, in *Journal of Indian Society of Periodontology*. 2011. p. 205.
110. Mandeville, F.B., *Aloe vera in the treatment of radiation ulcers of mucous membranes*. Radiology, 1939. **32**: p. 598-599.
111. Davis, R.H., et al., *Anti-inflammatory and wound healing activity of a growth substance in Aloe vera*. J Am Podiatr Med Assoc, 1994. **84**(2): p. 77-81.
112. U.S. Food and Drug Administration, D.o.H.a.H.S., *Gengigel(R) Premarket notification K053342*. 2007.
113. Baisse, E., Piotrowski, B., Piantoni, P., Brunel, G., *Results of an experimental study: Action of hyaluronic acid on the wound healing process following extraction* Clinical Pathology, 2003.
114. Shuvaev, V.V., et al., *Streptavidin-biotin crosslinking of therapeutic enzymes with carrier antibodies: nanoconjugates for protection against endothelial oxidative stress*. Methods Mol Biol, 2004. **283**: p. 3-19.
115. Sano, T. and C.R. Cantor, *Cooperative Biotin Binding by Streptavidin - Electrophoretic Behavior and Subunit Association of Streptavidin in the Presence of 6-M Urea*. Journal of Biological Chemistry, 1990. **265**(6): p. 3369-3373.
116. Diamandis, E.P. and T.K. Christopoulos, *The Biotin (Strept)Avidin System - Principles and Applications in Biotechnology*. Clinical Chemistry, 1991. **37**(5): p. 625-636.
117. Gonzalez, M., C.E. Argarana, and G.D. Fidelio, *Extremely high thermal stability of streptavidin and avidin upon biotin binding*. Biomolecular Engineering, 1999. **16**(1-4): p. 67-72.
118. Patel, M.M., et al., *Mucin/poly(acrylic acid) interactions: A spectroscopic investigation of mucoadhesion*. Biomacromolecules, 2003. **4**(5): p. 1184-1190.

119. Gokce, K., Benderli, Y., *Antimicrobial action of various polyacrylic acids on streptococcus mutans and actinomyces viscosus*. Oral Health & Dental Management, 2003. **2**(4): p. 42-46.
120. Bou-Gharios, G., et al., *Extra-cellular matrix in vascular networks*. Cell Prolif, 2004. **37**(3): p. 207-20.
121. Parkes, R.J. and S.L. Hart, *Adhesion molecules and gene transfer*. Advanced Drug Delivery Reviews, 2000. **44**(2-3): p. 135-152.
122. Wu, J.M., et al., *Physiochemical and biological properties of modified collagen sponge from porcine skin*. Journal of Wuhan University of Technology-Materials Science Edition, 2009. **24**(4): p. 619-626.
123. Cravatt, B.F., Y.S. Liu, and M.P. Patricelli, *Activity-based protein profiling: The serine hydrolases*. Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(26): p. 14694-14699.
124. Perez-Sala, D., et al., *15-deoxy-Delta (12,14)-prostaglandin J(2) inhibition of NF-kappa B-DNA binding through covalent modification of the p50 subunit*. Journal of Biological Chemistry, 2001. **276**(38): p. 35530-35536.
125. Besselink, G.A.J., T. Beugeling, and A. Bantjes, *N-Hydroxysuccinimide-Activated Glycine-Sepharose - Hydrolysis of Activated Groups and Coupling of Amino-Compounds*. Applied Biochemistry and Biotechnology, 1993. **43**(3): p. 227-246.
126. Staros, J.V., R.W. Wright, and D.M. Swingle, *Enhancement by N-Hydroxysulfosuccinimide of Water-Soluble Carbodiimide-Mediated Coupling Reactions*. Analytical Biochemistry, 1986. **156**(1): p. 220-222.
127. Timkovich, R., *Detection of Stable Addition of Carbodiimide to Proteins*. Analytical Biochemistry, 1977. **79**(1-2): p. 135-143.
128. Grabarek, Z. and J. Gergely, *Zero-Length Crosslinking Procedure with the Use of Active Esters*. Analytical Biochemistry, 1990. **185**(1): p. 131-135.
129. Haddleton, D.M., et al., *Biotin Functionalized Poly(sulfonic acid)s for Bioconjugation: In Situ Binding Monitoring by QCM-D*. Journal of Polymer Science Part a-Polymer Chemistry, 2011. **49**(5): p. 1163-1173.
130. Green, N.M., *A Spectrophotometric Assay for Avidin and Biotin Based on Binding of Dyes by Avidin*. Biochemical Journal, 1965. **94**: p. 23C-24C.

131. Lee, J.Y., et al., *Comparison of the composition of oral mucosal residual saliva with whole saliva*. Oral Diseases, 2007. **13**(6): p. 550-4.
132. *Pronase: Nuclease-free, isolated from Streptomyces griseus*. Roche Applied Science 2004; Version 4: Available from:
<http://www.biocity.cn/Shop/UploadPhotos/200605/20060521214743912.pdf>.
133. *Protease from Streptomyces griseus: Product descripton*. Sigma Aldrich.
134. Inoue, H., Ono, K., Masuda, W., Inagaki, T., Yokota, M., and Inenaga, K., *Rheological properties of human saliva and salivary mucins*. Journal of Oral Biosciences, 2008. **50**(2): p. 131-141.
135. *Instructions: Pierce(R) Iodination Reagent*. Thermoscientific Available from:
<http://www.piercenet.com/instructions/2160485.pdf>.
136. Kho, H.S., et al., *Comparison of the composition of oral mucosal residual saliva with whole saliva*. Oral Diseases, 2007. **13**(6): p. 550-554.
137. StatSoft, I., *Electronic Statistics Textbook*. 2011: Tulsa, OK.
138. Minitab. *Technical Support Document: Multiple Comparison Methods*. 1-9]. Available from:
<http://www.minitab.com/support/documentation/Answers/Multiple%20Comparison%20Methods.pdf>.
139. Grafen, A., Hails, R, *Minitab Supplement to accompany Modern Statistics for the Life Sciences*. 2002.
140. Kim, J., Yoon, J., *Protein adsorption on polymer particles*. Encyclopedia of Science and Colloid Science, 2002: p. 4373-4380.
141. Andrade, J.D., V. Hlady, and A.P. Wei, *Adsorption of Complex Proteins at Interfaces*. Pure and Applied Chemistry, 1992. **64**(11): p. 1777-1781.
142. Krozer, A., K. Reimhult, and K. Petersson, *QCM-D analysis of the performance of blocking agents on gold and polystyrene surfaces*. Langmuir, 2008. **24**(16): p. 8695-8700.
143. Mielczarski, J.A., et al., *Efficiency of blocking of non-specific interaction of different proteins by BSA adsorbed on hydrophobic and hydrophilic surfaces*. Journal of Colloid and Interface Science, 2010. **341**(1): p. 136-142.

144. Nimeri, G., et al., *Adsorption of Fibrinogen and Some Other Proteins from Blood-Plasma at a Variety of Solid-Surfaces*. Journal of Biomaterials Science-Polymer Edition, 1994. **6**(6): p. 573-583.
145. Christiano, S.P. and K.C. Fey, *Silicone antifoam performance enhancement by nonionic surfactants in potato medium*. Journal of Industrial Microbiology & Biotechnology, 2003. **30**(1): p. 13-21.
146. Svensson, O., et al., *Layer-by-layer assembly of mucin and chitosan - Influence of surface properties, concentration and type of mucin*. Journal of Colloid and Interface Science, 2006. **299**(2): p. 608-616.
147. Picart, C., et al., *Buildup mechanism for poly(L-lysine)/hyaluronic acid films onto a solid surface*. Langmuir, 2001. **17**(23): p. 7414-7424.
148. Lavalley, P., et al., *Free standing membranes made of biocompatible polyelectrolytes using the layer by layer method*. Journal of Membrane Science, 2005. **253**(1-2): p. 49-56.
149. Meyer, W., K. Neurand, and B. Radke, *Collagen Fiber Arrangement in the Skin of the Pig*. Journal of Anatomy, 1982. **134**(Jan): p. 139-148.
150. Izumi, K., et al., *Intraoral grafting of an ex vivo produced oral mucosa equivalent: a preliminary report*. International Journal of Oral and Maxillofacial Surgery, 2003. **32**(2): p. 188-197.
151. King, C.A., *A general study on the adsorption of protein by tissue*. Biochimica Et Biophysica Acta, 1968. **154**(2): p. 269-77.
152. Jensen, E.V., *Sulfhydryl-Disulfide Interchange*. Science, 1959. **130**(3385): p. 1319-1323.

VITA

Date and place of birth:

- January 14th 1986, Chennai, India.

Educational institutions attended and degrees already awarded:

- B.Tech., Bachelors in Chemical Engineering,
Alagappa College of Technology, Anna University, Chennai, India.

Professional positions held

- Teaching Assistant & Research Assistant, Department of Chemical & Materials Engineering, University of Kentucky, USA.

Typed name of student on final copy

- Sundar Prasanth Authimoolam